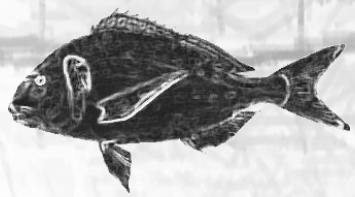
Estudio de los patrones de expresión de genes implicados en la síntesis de ácidos grasos de cadena muy larga durante el desarrollo de la dorada y el lenguado, y su regulación nutricional

Tesis Doctoral

Miguel Torres Rodríguez



Diciembre-2020

Dirigida por: Juan C. Navarro Tárrega y Francisco Hontoria Danés

VNIVERSITAT POLITÈCNICA VALÈNCIA

IATS INSTITUTO DE ACUICULTURA TORRE DE LA SAL

CSIC CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

Universidad Politécnica de Valencia

Instituto de Acuicultura de Torre de la Sal
(**IATS-CSIC**)

Estudio de los patrones de expresión de genes implicados en la síntesis de ácidos grasos de cadena muy larga durante el desarrollo de la dorada y el lenguado, y su regulación nutricional

Memoria presentada por **Miguel Torres Rodríguez** para optar al Grado de Doctor

Tesis doctoral dirigida por:

Juan Carlos Navarro Tárrega

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Fdo. Miguel Torres Rodríguez

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Para desarrollar el trabajo de investigación descrito en esta memoria, Miguel Torres Rodríguez recibió una beca predoctoral de la Excma. Diputación de Castellón.

A mis abuelos…

A todos los animales de investigación…

Recuerdo cuando hace algunos años, unos meses antes de enfrentarnos al examen de selectividad, un grupo de amigos dudábamos entre irnos al bar a echar unas cervezas o entrar a recibir alguna charla sobre carreras universitarias. Obviamente, como suele ocurrir en la mente de cualquier adolescente, ganó la primera opción. Sin embargo, en nuestro camino algo llamó mi atención. Un cartel donde aparecía un ratón blanco se interpuso entre mí y el bar. Mi vocación por los animales era de sobra conocida, y esa imagen me hizo cambiar de opinión. Entré a la charla sobre el Grado en Biología. Esa decisión no solo supuso el inicio de mi etapa universitaria, sino que ha hecho posible que hoy me encuentre escribiendo estas líneas.

Mentiría si digo que toda esta etapa ha sido un camino de rosas. Pero sin duda ha sido un camino de aprendizaje. No solo a nivel académico y profesional, sino personal. Comencé mi etapa predoctoral creyéndome que me iba a comer el mundo y que sabía más que nadie. La acabo haciendo referencia a la frase más célebre de Sócrates "solo sé que no se nada". Sin duda me queda un gran camino que recorrer, pero en este pasito dado, tengo varias cosas que agradecer.

A mis directores de Tesis, el Dr. **Juan Carlos Navarro** y el Dr. **Francisco Hontoria**. No solo, por haberme dado la oportunidad de introducirme en el mundo de la investigación y de la acuicultura, si no por saber guiar mi carácter fuerte hasta

el final. Obviamente, aunque suene muy pelota, sin ellos no podría haber llevado a cabo esta tesis.

También me gustaría acordarme del Dr. **Oscar Monroig**, pues, aunque me daba mucho coraje ver todo el manuscrito en rojito tras su revisión de un artículo, tengo que reconocer que siempre era para mejorarlo.

No puedo olvidarme de mis compañeras de la asociación protectora de animales de Oropesa del Mar, **La Morada Animalista**. Con ellas puede canalizar y aplicar mi amor y respeto por los animales, especialmente por aquellos más desfavorecidos. Juntos hemos rescatado, curado, alimentado y buscado un hogar a decenas de gatitos. En esta asociación he conocido a grandes personas, con corazones aún más grandes, que no dudaban ni un segundo en prestar ayuda tanto a personas como a animales. Sin duda, una de las personas con mayor bondad que he tenido la suerte de conocer ha sido **Lucilo.** De él he aprendido que el dinero no da la felicidad, y que con muy poquito, si se tienen ganas e ilusión, se pueden hacer grandes cosas. Sin duda, su pérdida dejó huérfanos a muchos animalitos y un gran vacío en la asociación. De él me llevo el mejor recuerdo que puedo tener, mi perrito abuelete **Nano**.

Durante estos cuatro años ha habido una persona que ha sido mi gran pilar. La persona que ha aguantado mis idas y venidas, mis días buenos y no tan buenos,

mis “tranquila que este animal no nos lo quedamos” y hemos acabado con un acuario lleno de peces, dos ratas, una tortuga, tres gatos (mis niños), un perro y algún pájaro ocasional. **Rocío**. Mi pareja y compañera de viaje. Ese monillo loco que no solo me hace vivir, sino sentir. Sin duda, nos quedan muchas aventuras por disfrutar juntos.

No puedo evitar pensar en **mi familia**. Especialmente en **mis abuelos**. Ellos han sido siempre un ejemplo de esfuerzo, constancia y sacrificio. Siempre me han dicho “quien algo quiere, algo le cuesta”. Desgraciadamente, mi abuela Antonia no va a poder felicitarme por culminar esta etapa. Pero no es necesario, pues sé que ella siempre estará orgullosa de su nieto.

Cómo no mencionar a los grandes responsables de mi vocación por el mundo animal. Todos esos bichitos de cuatro patas que, aunque sin saberlo, han hecho de mi día a día algo especial. No hay nada más reconfortarte que llegar a tu casa y que una bolita de pelo, ajeno a todas las circunstancias, te reciba restregando su hocico contra ti, ronroneando, o acurrucándose a tu lado mientras duermes. Algunos sólo llegaron de paso, pero dejando una gran zarpa en mi corazón. **Caloret**, mi gatita fallera. **Rulo**, mi bebé grandote. Otros llegaron para quedarse y hoy forman parte de nuestra gran familia. **Catijo**, mi compañero incondicional. **Calcetines**, la ternura con bigotes. **Philips**, mi vaquita regordeta. **Nano**, mi abuelete hipocondríaco. Como no acordarse de **Berenjeno**, mi gatito soscón. **Toby**, mi saco de nervios. Por último, aunque no menos importante, **Lagartijo**, el gran responsable de que exista esta gran familia.

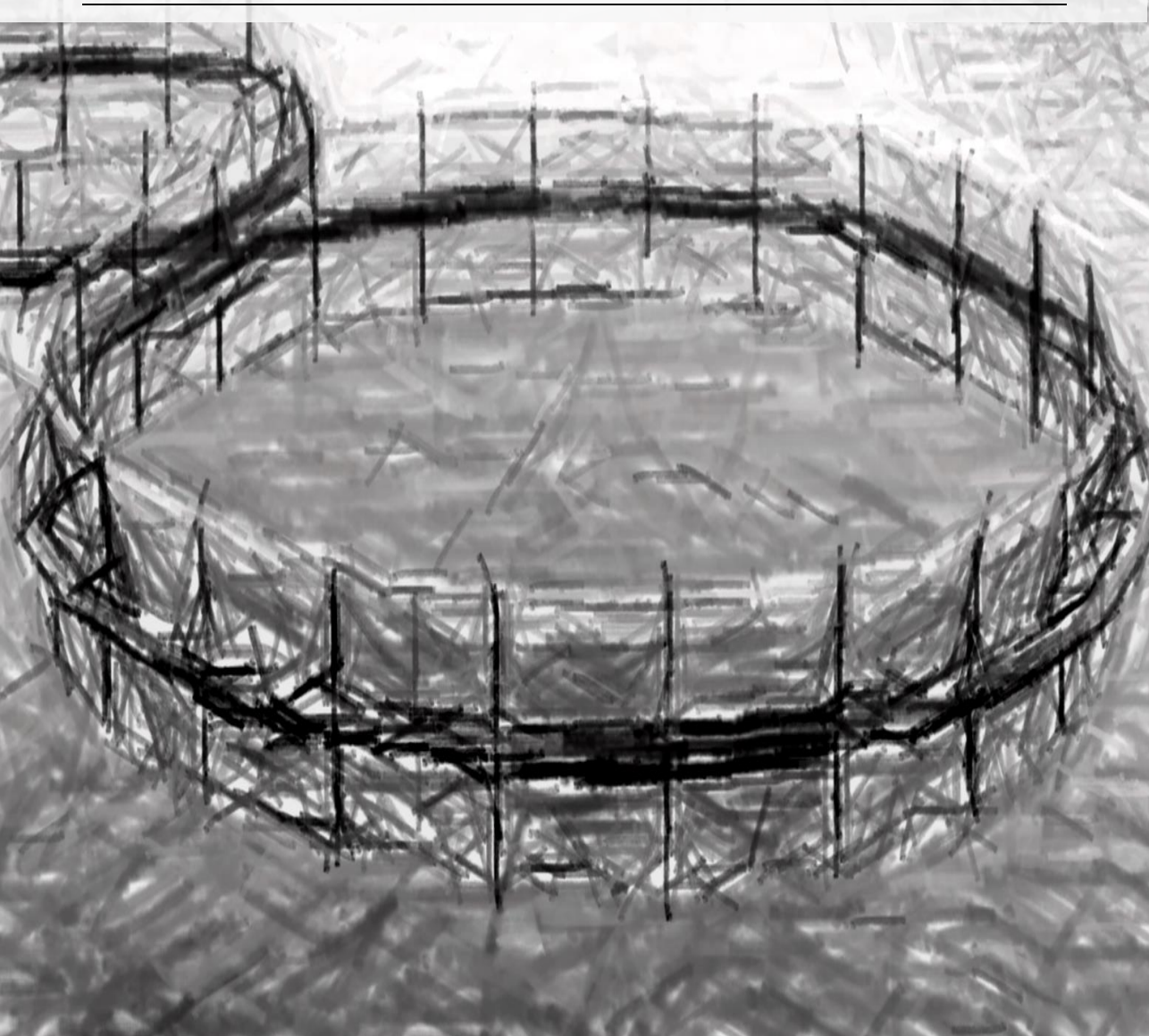
Aunque sin duda, el reconocimiento de esta tesis doctoral debe ser para las decenas de larvas, poslarvas y adultos de **dorada** y **lenguado senegalés** que, sin ser conocedores ni prestar su opinión, han contribuido a engrandecer la palabra CIENCIA. Todas las páginas de este trabajo de investigación sobrarían si no hubiese sido por ellos. Como muestra de gratitud, me gustaría dedicar esta tesis no sólo a ellos, sino a todos los animales que día a día hacen posible que la ciencia avance. Debemos tratarlos con respeto, garantizar su bienestar, formarnos continuamente para su mejor manejo y, sin duda, asegurarnos de que algún día su presencia no sea necesaria para el correcto avance de la ciencia. No sé si ese día llegará. Pero mientras tanto, solo me queda ofrecer mi respeto y agradecimiento a los **animales de investigación**. Esta tesis va dedicada a todos ellos.

"*El amor por todas las criaturas vivientes es el más noble atributo del ser humano"*

(Charles Darwin)

CAPÍTULO 1:

Introducción



1.1. Estado actual de la acuicultura

Históricamente, el mar ha sido considerado una fuente inagotable de recursos pesqueros. No fue hasta la década de 1950 cuando la acuicultura empezó a desarrollarse, propiciada por la disminución de las poblaciones naturales de peces y el creciente interés de la población por los productos acuícolas.

La acuicultura se define como "la producción en el agua de animales y plantas mediante técnicas encaminadas a hacer un uso más eficiente de los recursos naturales" (APROMAR, 2019). De orígenes asiáticos, y aunque no exenta de controversia, la acuicultura ha experimentado un fuerte aumento en las últimas décadas a nivel global, siendo una de las actividades de producción de alimentos con mayor proyección. De ahí, que la producción acuícola mundial se supere año tras año, alcanzando en 2017 los 111,9 millones de t, lo cual supone un 3,5 % más que en 2016. Ese mismo año, la acuicultura superó a la producción pesquera (pesca extractiva) en 18,3 millones de t (**Figura 1.1**), alcanzando un valor de 199,6 mil millones de euros en primera venta (APROMAR, 2019).

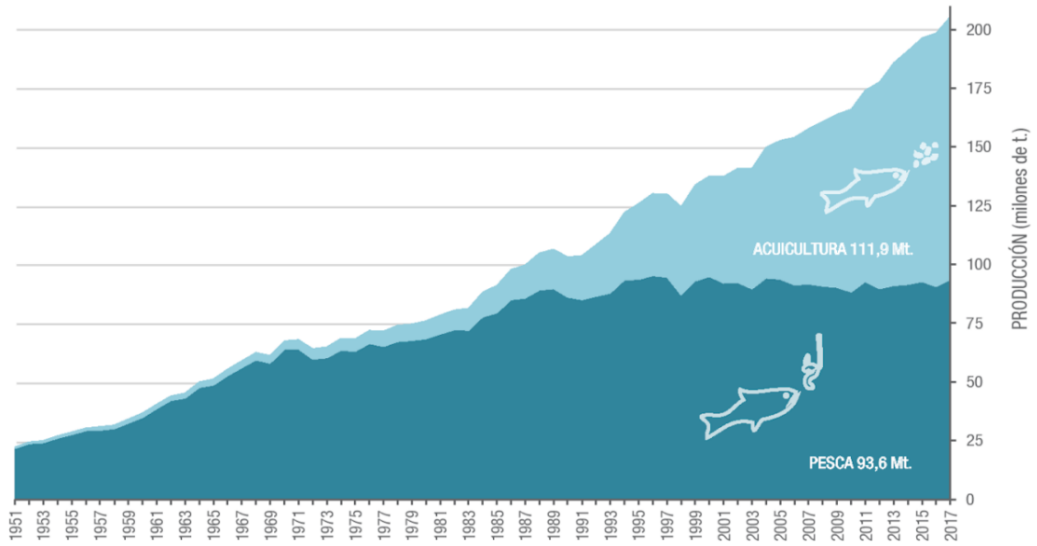


Figura 1.1. Evolución de la producción acuática mundial (acuicultura más pesca) en el periodo 1950-2017. Imagen extraída del informe APROMAR 2019 (Datos FAO).

Actualmente, la sociedad humana se enfrenta al gran desafío de tener que proporcionar alimentos y medios de vida a una población que, para mediados del siglo XXI, superará con creces los nueve mil millones de personas, al tiempo que deberá afrontar los efectos del cambio climático y de la degradación ambiental en la base de los recursos (FAO, 2020). Por ello, cada vez son más los estudios que colocan a los recursos acuícolas, y por ende a la acuicultura, como una estrategia a reforzar, para conseguir los Objetivos de Desarrollo Sostenible (ODS) establecidos por la Organización de las Naciones Unidas (ONU) en la Agenda 2030. Entre ellos se encuentran: erradicar la pobreza y el hambre, mejorar las políticas de empleo e igualdad de género, mejorar la producción y el consumo de alimentos, combatir el

cambio climático, así como preservar los recursos y los ecosistemas marinos (APROMAR, 2019). Para tal fin, desde el sector acuícola se pretende avanzar en la reformulación de políticas, en la planificación y en la gestión del desarrollo sostenible, permitiendo implementar estrategias alimentarias basadas en una dieta rica en productos acuícolas, que pueden contribuir sustancialmente a la mejora de la nutrición y la seguridad alimentaria a nivel global (APROMAR, 2019; Hicks *et al*., 2019).

Con el objetivo de alcanzar los ODS marcados por la ONU, desde la Unión Europea (UE) se ha desarrollado el concepto de "Crecimiento Azul". Este concepto surge como una estrategia a largo plazo de apoyo al crecimiento sostenible de los sectores marino y marítimo, permitiendo así el reconocimiento de los mares/océanos como impulsores de la economía europea. La economía azul emplea a más de 5,4 millones de personas en la UE y supone un valor añadido bruto de casi 500 mil millones de euros al año. Estos datos colocan a la UE como el primer y más relevante mercado de productos acuícolas a nivel mundial (APROMAR, 2019).

En 2018, la UE registró un consumo de 13 millones de t de productos acuícolas, de las cuales 9,5 millones fueron importadas (APROMAR, 2019). Uno de los objetivos de la UE es mejorar la autosuficiencia de productos acuícolas, siendo actualmente de tan solo el 27,4 %. (APROMAR, 2019). Es aquí donde la acuicultura

juega un papel clave, situándose como un apoyo esencial del “Crecimiento Azul" que adoptan la UE y España.

En 2018 la UE registró un total de 1.364.400 t de producto de acuicultura frente a las 1.353.201 t registradas en 2017 (FAO, 2020). Estos datos suponen un aumento del 5,6 % y del 0,8 % en cuanto a 2016 y 2017, respectivamente (APROMAR, 2019; FAO, 2020). En 2017, dicha producción alcanzó un valor de 4.147 millones de euros, aumentando un 11,3 % el valor registrado en 2016 (APROMAR, 2019). Sin embargo, la importancia de la acuicultura difiere entre los países miembros de la UE, siendo España el estado miembro con mayor volumen de producción acuícola, con más 311 mil t registradas en 2017 (**Figura 1.2**), de las que 66.591 t corresponden a pescado (APROMAR, 2019).

Estas cifras colocan a España como el tercer país productor de pescado de la UE, con un 9,1 % del total y un crecimiento anual del 3,9 %, alcanzando un valor de 331,7 millones de euros, lo que supone el 10,2 % del valor total de la piscicultura de la UE (APROMAR, 2019).

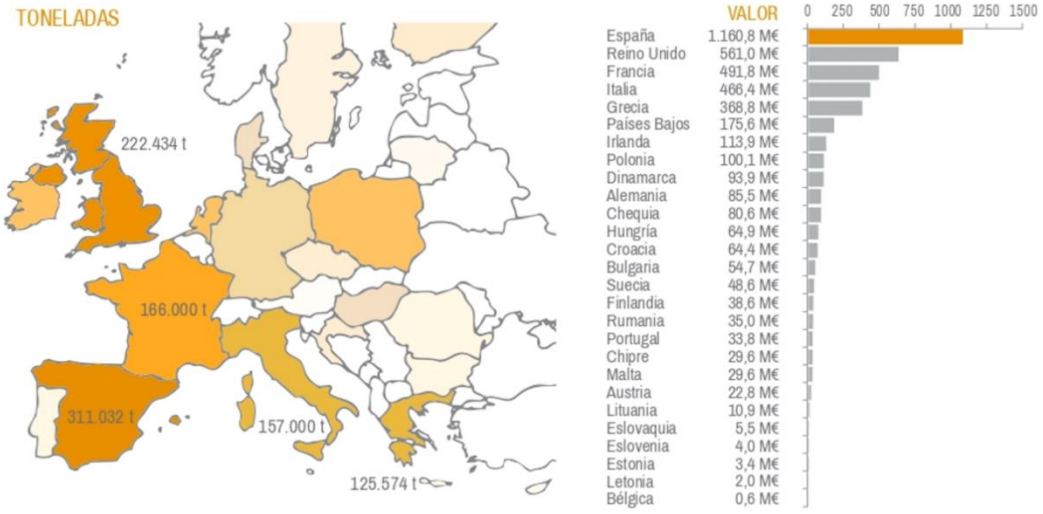


Figura 1.2. Distribución de la producción de acuicultura en los Estados miembros de la Unión Europea por su cantidad (t) y valor (millones de euros) en 2017. Imagen extraída del informe APROMAR 2019 (Datos FAO).

De entre las especies de peces producidas en España, la lubina, la trucha arco iris y la dorada son las especies con mayor volumen de producción, registrando en 2018 unos valores de 22.460, 18.856 y 14.930 t, respectivamente (**Figura 1.3**).

Otra de las cifras que refleja la importancia del sector de la acuicultura en España es la tasa de empleo, estimándose, según datos del Ministerio de Agricultura, Pesca y Alimentación (MAPA), en 40.378 puestos laborales en todo el territorio nacional, de los cuales, 16.151 son directos (APROMAR, 2019).

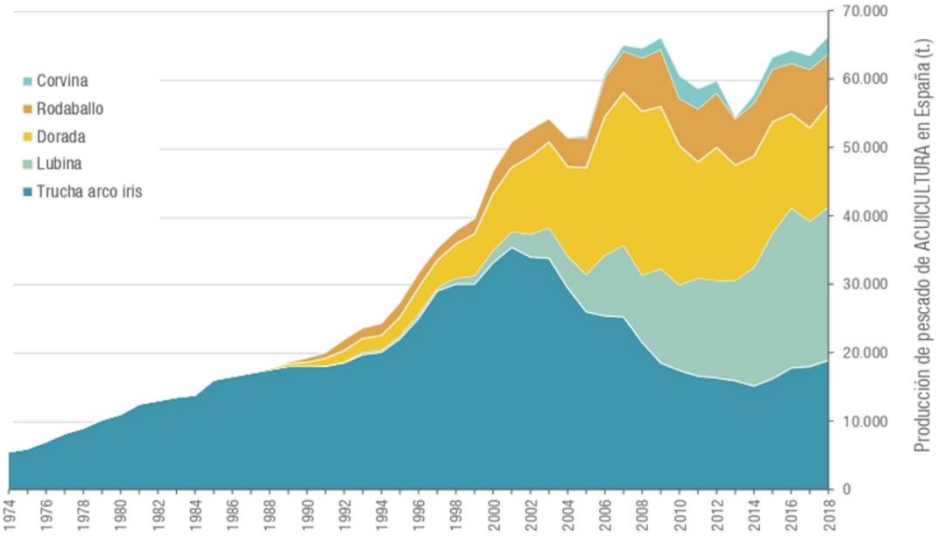


Figura 1.3. Evolución de la producción de peces de acuicultura en España, en t, para las principales especies en el periodo 1975-2018. Imagen extraída del informe APROMAR, 2019 (Datos MAPA y APROMAR).

1.2. La dorada (*Sparus aurata*)

La dorada (*Sparus aurata*), es un teleósteo perciforme perteneciente a la familia Sparidae. Morfológicamente, presenta un cuerpo ovalado comprimido lateralmente. Uno de los caracteres identificativos de la especie es la presencia de una banda cefálica de color amarillo situada entre los ojos. Esta franja, sobre la que aparece otra banda de color negro, es más patente en individuos adultos. Otra característica distintiva es la presencia de una mancha negra ubicada sobre el opérculo branquial, al inicio de la línea lateral (**Figura 1.4**.) (Ortega, 2008). Presenta una distribución subtropical, encontrándose de manera natural en el mar

Mediterráneo, las costas del Atlántico Este desde Gran Bretaña a Senegal y, en menor medida, en el mar Negro (Koven, 2002). De hábitos eurihalinos y euritérmicos, puede tolerar un rango de salinidad entre 3 y 70 ‰, así como temperaturas de entre 7 y 33 ºC (Ortega, 2008). Ese amplio rango de tolerancia termo-salina nos permite encontrarla tanto en ambientes marinos, como salobres tales como lagunas costeras y áreas estuarinas, habitando estas zonas particularmente durante las primeras etapas de su ciclo de vida (Cultured Aquatic Species Information Programme, FAO).

Su dieta es eminentemente carnívora, alimentándose de moluscos bivalvos, pequeños peces y/o cefalópodos. Además, puede practicar la herbivoría de manera puntual (Koven, 2002).

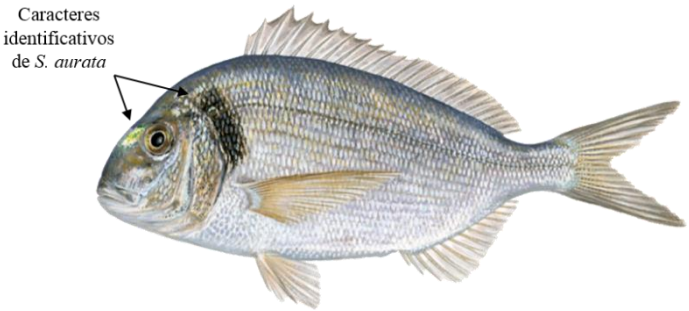


Figura 1.4. Dorada (*Sparus aurata*). Adaptación de la imagen obtenida en http://guiadepescado.wwf.es.

La principal característica reproductiva de esta especie es su hermafroditismo protándrico. Durante los dos primeros años de vida, todos los individuos se desarrollan como machos funcionales. En esta fase masculina, la gónada bisexual presenta espermatogénesis asincrónica y áreas ováricas no funcionales. Una vez transcurridos los dos primeros años de vida y alcanzado un tamaño de unos 30 cm, se produce el desarrollo ovárico, también asincrónico, dando lugar a hembras reproductoras capaces de desovar entre 20 y 80 mil huevos diarios durante el periodo reproductivo. En cautiverio, el cambio de sexo está condicionado por factores sociales y hormonales (Cultured Aquatic Species Information Programme, FAO; Koven, 2002).

Su cultivo, que está condicionado por diferentes factores, puede hacerse de manera extensiva en lagunas costeras o estanques, intensiva en instalaciones terrestres y/o jaulas marinas (**Figura 1.5**), o semi-intensivo combinando ambos métodos.

La producción europea (Turquía incluida) de juveniles de dorada en 2018 fue de 734.299 millones de unidades, de los que 37,5 millones, corresponden a España. La producción nacional se concentra en la Comunidad Valenciana (59 %), Cantabria (22 %), Islas Baleares (23 %) y Andalucía (9 %).

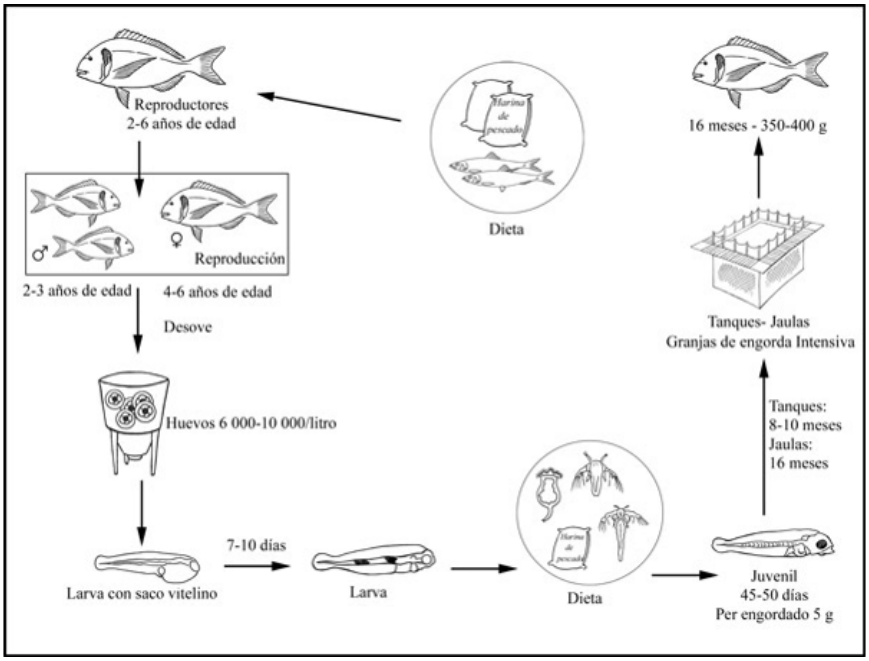


Figura 1.5. Ciclo de producción intensivo de *Sparus aurata* (Cultured Aquatic Species Information Programme, FAO). Adaptación de la imagen obtenida en www.fao.org.

En el mercado nacional, una vez alcanzado el tamaño adecuado para consumo, su precio en primera venta en 2018 fue de 4,41 euros/kg, valorándose en 65,8 millones de euros el total de las 14.930 t producidas.

Aunque el consumo de dorada en los hogares españoles en 2018 se incrementó un 5 %, el precio por kg se vio reducido en un 9,5 % con respecto a 2017 (APROMAR, 2019). Actualmente el mercado convencional se encuentra saturado, por lo que la industria de la acuicultura mediterránea necesita actualizar los métodos de producción y comercialización para mantener su rentabilidad. Así, el desarrollo

de mercados alternativos y de productos con valor añadido, como el cultivo ecológico de peces y/o de mayor tamaño para facilitar su procesado, parece ser una tendencia al alza (Cultured Aquatic Species Information Programme, FAO).

1.3. El lenguado senegalés (*Solea senegalensis*)

El lenguado senegalés (*Solea senegalensis*) es un pez plano perteneciente a la familia Soleidae. Presenta un cuerpo ovalado y asimétrico, manteniendo los ojos sobre el costado derecho (cara ocular). Su cara ciega presenta una tonalidad blanquecina, mientras la cara ocular presenta varias tonalidades de pigmentación marrón, pudiendo ésta presentar pequeñas manchas de diferente tonalidad distribuidas por todo el cuerpo. Uno de los caracteres morfológicos diferenciadores en esta especie es la pigmentación de la aleta pectoral presente en el lado ocular, ya que en *Solea senegalensis* su membrana interradial es de color negro, mientras que en *Solea solea* (lenguado común) dicha aleta presenta una mancha negra compacta en su mitad posterior (**Figura 1.6**) (Rodríguez y Peleteiro, 2014).

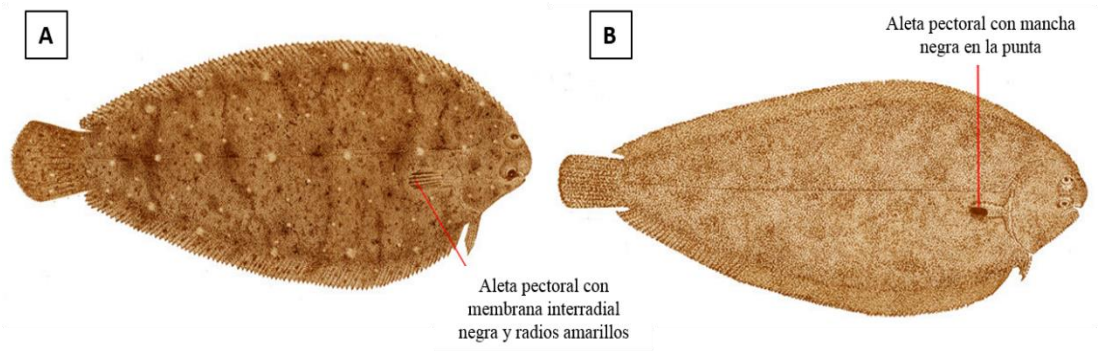


Figura 1.6. *Solea senegalensis* (A) y *Solea solea* (B). Diferente pigmentación de la aleta pectoral del lado ocular según la especie. Adaptación de las imágenes disponibles en www.IctioTerm.es.

Su distribución natural abarca el Atlántico, desde el Golfo de Vizcaya hasta las costas de Senegal, siendo poco frecuente en el Mediterráneo Occidental. Dependiendo de la región geográfica donde se encuentre, puede llegar a medir 60 cm de longitud, en el caso del Atlántico, y 45 cm, en el caso del Mediterráneo, y alcanzar un peso de 3 kg, siendo las hembras de mayor tamaño que los machos (Rodríguez y Peleteiro, 2014). Su alimentación es carnívora, alimentándose de pequeños invertebrados bentónicos, larvas de poliquetos, moluscos bivalvos y pequeños crustáceos (Cañavate, 2013).

Se trata de especie dioica, sin un dimorfismo sexual acentuado. Su desarrollo sexual se lleva a cabo al alcanzar los tres años de edad y un tamaño aproximado de 30 cm. Tras la puesta, llevada a cabo entre los meses de marzo a junio, las larvas recién eclosionadas presentan un carácter pelágico hasta los 10-12 días de vida,

cuando experimentan una metamorfosis de una semana de duración, aproximadamente, tras la que las larvas posmetamórficas desarrollan un carácter bentónico (**Figura 1.7**) (Cañavate, 2013).

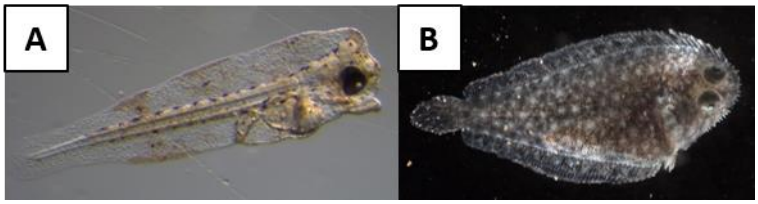


Figura 1.7. Larva premetamórfica (**A**) y posmetamórfica (**B**) de *Solea senegalensis*.

Si bien *S. senegalensis* suscita un gran interés piscícola y comercial, su cultivo está aún por desarrollar, pues, aunque en la última década, gracias a los diferentes programas de investigación, se ha avanzado mucho desde el punto de vista científico, técnico y productivo, aún existen aspectos relacionados con su reproducción y control sanitario en cautiverio que hacen que el despegue de su producción a nivel industrial se encuentre estancado (Cañavate, 2013; Howell y Dinis, 2019). Actualmente, aunque el ciclo integral está cerrado, su producción está centrada en el engorde de alevines en instalaciones terrestres con sistemas de recirculación que permiten mantener una temperatura óptima de crecimiento que oscila entre 18-20 ºC (**Figura 1.8**) (Rodríguez y Peleteiro, 2014).

En 2018, su producción mundial fue de 1.616 t, de las que 774 t fueron producidas en España, principalmente en Galicia y Andalucía. Estas cifras suponen una regresión del 3,9 % a nivel mundial y del 6,7 a nivel nacional con respecto a las cifras registradas en 2017 (APROMAR, 2019).

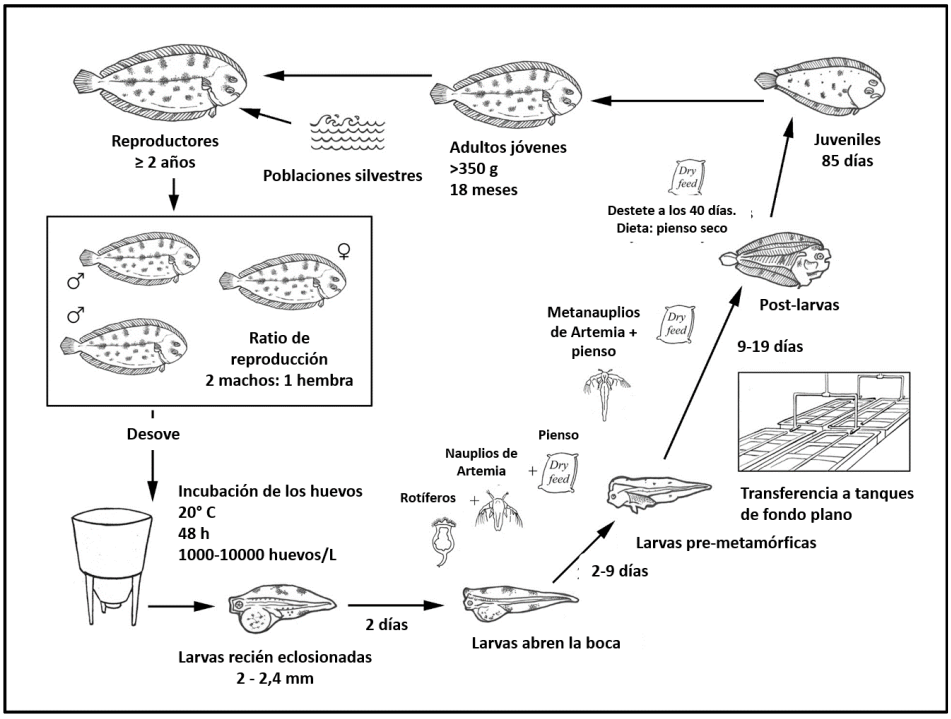


Figura 1.8. Ciclo de producción de *Solea senegalensis* (Cultured Aquatic Species Information Programme, FAO). Adaptación de la imagen obtenida en www.fao.org.

1.4. Lípidos

Los lípidos son un grupo de moléculas orgánicas de naturaleza hidrofóbica. Presentan una gran heterogeneidad estructural y funcional, lo que ha favorecido la existencia de diferentes sistemas de clasificación. En base a su polaridad, se pueden clasificar en lípidos neutros y polares. Los lípidos neutros o grasas simples, de naturaleza hidrofóbica, están constituidos por ésteres de ácidos grasos y alcohol, estando únicamente compuestos por carbono, hidrógeno y oxígeno (**Figura 1.9**). En la naturaleza podemos encontrarlos en forma de ceras con función estructural y de acilgliceroles con función de reserva energética, formando parte del tejido adiposo. Los lípidos polares, al igual que los neutros, están formados mayoritariamente por átomos de carbono, hidrógeno y oxígeno. Sin embargo, éstos contienen una región polar en cuya estructura pueden existir átomos de fósforo, azufre y/o nitrógeno (**Figura 1.10**). Los lípidos polares, de naturaleza anfipática, tienen una función predominantemente estructural, formando parte esencial de las membranas celulares. Entre ellos podemos encontrar glicerofosfolípidos y esfingolípidos.

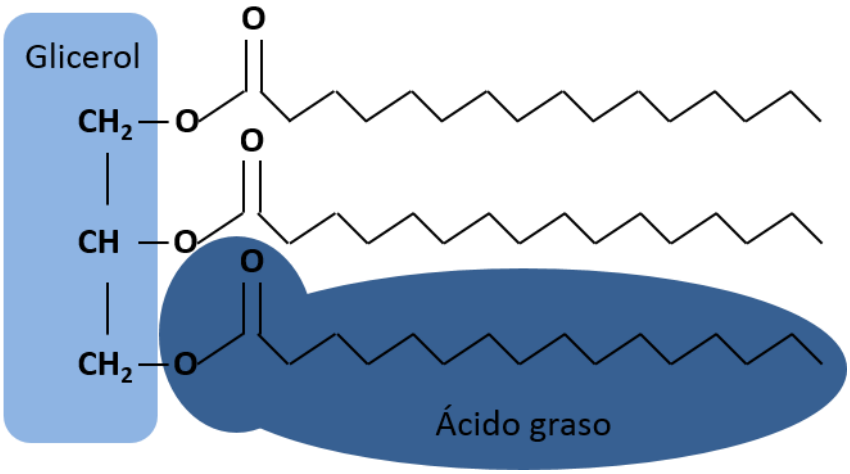


Figura 1.9. Estructura química general de un triglicérido (lípido neutro).

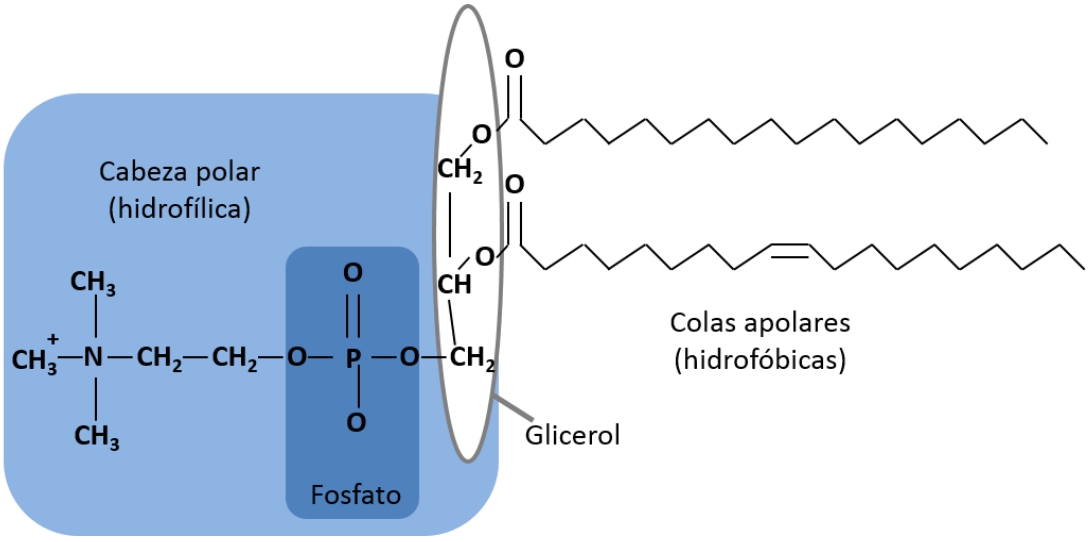


Figura 1.10. Estructura química general de un glicerofosfolípido (lípido polar).

En 2005, gracias al patrocinio del proyecto LIPID MAPS (www.lipidmaps.org), el Comité Internacional de la Clasificación y Nomenclatura de los Lípidos desarrolló el "Comprehensive Classification System for Lipids". Este sistema de clasificación está basado en la funcionalidad y características estructurales de los lípidos, diferenciando ocho grupos principales: ácidos grasos, glicerolípidos, glicerofosfolípidos, esfingolípidos, lípidos esteroles, lípidos prenoles, sacarolípidos y policétidos (Fahy *et al*., 2009).

Los glicerofosfolípidos y los esfingolípidos presentan un fuerte carácter anfipático, y son componentes estructurales de vital importancia para el mantenimiento de las membranas celulares. Los glicerofosfolípidos son derivados de la unión de un ácido fosfatídico (L-glicerol 3-fosfato esterificado en posición *sn-1* y *sn-2*) (**Figura 1.11**) y un grupo alcohol o aminoalcohol. El ácido fosfatídico se esterifica a las "bases" colina, etanolamina, serina, inositol y glicerol, formando los principales glicerofosfolípidos presentes en los tejidos animales: fosfatidilcolina, fosfatidiletanolamina, fosfatidilserina y fosfatidilinositol y fosfatidilglicerol (**Figura 1.12**).

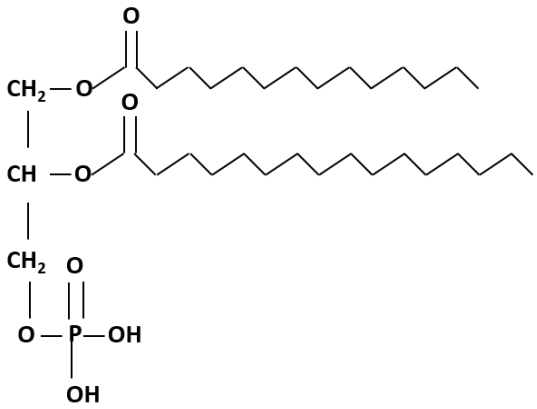


Figura 1.11. Ácido fosfatídico. Estructura básica de los glicerofosfolípidos.

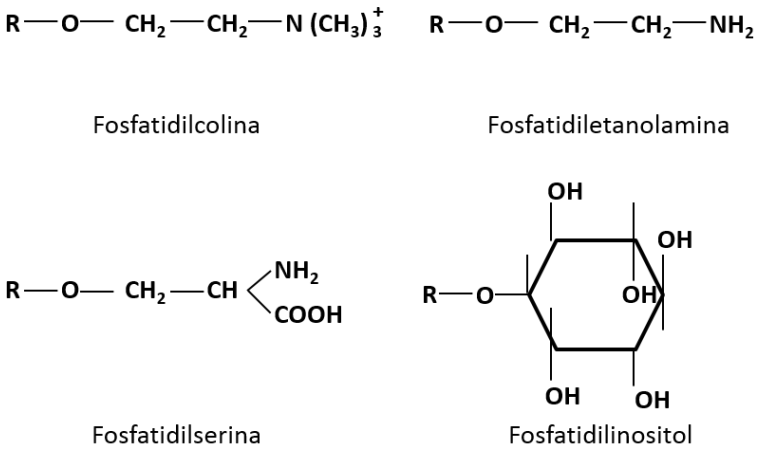


Figura 1.12. Fosfatidilcolina, fosfatidiletanolamina, fosfatidilserina y fosfatidilinositol. R, grupo fosfatidil.

Los esfingolípidos son derivados de la ceramida, molécula formada por esfingosina y un ácido graso saturado de cadena larga (en inglés *long-chain saturated fatty acid*; LC-SFA). En función del compuesto que se una al grupo

hidroxilo de la ceramida, éstos se clasifican en: esfingolípidos (ceramida unida a ácido fosfórico y alcohol), glucoesfingolípidos (ceramida unida a uno o más monosacáridos) y esfingomielina (ceramida unida a fosfocolina). La esfingomielina, que desempeña un papel clave en el mantenimiento de la integridad del tejido nervioso, es uno de los esfingolípidos en los que el grupo de fosfocolina se reemplaza por uno o más azúcares, incluyendo glucosa y galactosa, para formar glucoesfingolípidos como p. ej.: cerebrósidos y gangliósidos (Rodríguez-Barreto, 2014; Sargent *et al*., 2003).

Dentro de los denominados lípidos neutros, que presentan un fuerte carácter hidrofóbico, podemos encontrar: triglicéridos (**Figura 1.9**), diglicéridos y ésteres de esteroles como el colesterol (**Figura 1.13**) (Sargent *et al*., 2003). Los diglicéridos y triglicéridos, que actúan como sustratos energéticos, están constituidos por un glicerol esterificado con dos y tres ácidos grasos, respectivamente. Entre los esteroles, el colesterol es el mayoritario en los tejidos animales, incluidos los peces. Éste puede existir esterificado a un ácido graso, como es el caso de los ésteres de colesterol existentes en el plasma sanguíneo, o no esterificado, como ocurre en las membranas plasmáticas celulares (Sargent *et al*., 2003).

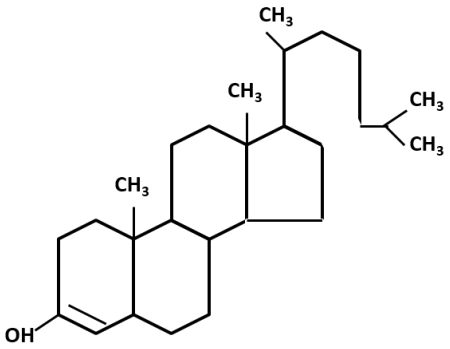


Figura 1.13. Colesterol.

A excepción de esteroles y prenoles, la mayoría de los lípidos presentan ácidos grasos en su estructura, ya sean esterificados a grupos alcohol, como en el caso de los glicéridos, o a grupos amino, como en el caso de los esfingolípidos. Los ácidos grasos se caracterizan por tener una estructura hidrocarbonada, de longitud variable y naturaleza hidrofóbica, con un extremo carboxilo terminal (**Figura 1.14**).

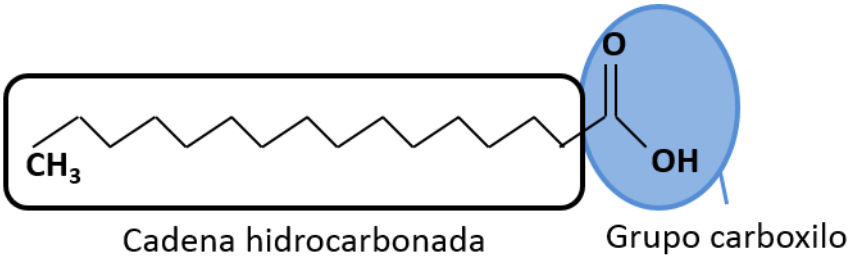


Figura 1.14. Estructura básica de un ácido graso.

1.5. Ácidos grasos: características y clasificación

Los ácidos grasos pueden clasificarse, según la presencia o no de insaturaciones en su cadena hidrocarbonada, en ácidos grasos saturados (en inglés *saturated fatty acids*; SFA), cuando no existe ninguna doble unión, o insaturados (en inglés *unsaturated fatty acids*; UFA), cuando existen dobles enlaces. A su vez, los ácidos grasos insaturados, en función del número de dobles enlaces estructurales que posean, se clasifican en monoinsaturados (en inglés *monounsaturated fatty acids*; MUFA) cuando poseen un único doble enlace, o poliinsaturados (en inglés *polyunsaturated fatty acids*; PUFA) cuando poseen varios dobles enlaces estructurales.

Asimismo, basándonos en la longitud de su cadena hidrocarbonada, su grado de insaturación o número de dobles enlaces existentes y la posición de éstos, existen diferentes formas de nombrar a los ácidos grasos. El método más extendido es el sistema de nomenclatura abreviado "n" u "omega (ω)". Este sistema se basa en el número de insaturaciones existente y en la posición que el primer doble enlace ocupa en la cadena hidrocarbonada partiendo del extremo metilo terminal. Así, los ácidos grasos se designan con una notación numérica de tres cifras. El primer número indica el número de átomos de carbono que conforman la cadena hidrocarbonada. El segundo número, separado del primero por dos puntos, corresponde al número de insaturaciones presente en dicha cadena. El tercer número, que aparece separado por

las letras "n" u "ω", hace referencia a la posición de la primera insaturación contando a partir del grupo metilo. De esta manera, un ácido graso con 20 átomos de carbono, sin ninguna insaturación, se designa 20:0. Si este mismo ácido graso presentase insaturaciones en su estructura, se designaría, tal como ha sido comentado anteriormente, según el número de éstas y la posición del primer doble enlace, partiendo del extremo metilo terminal. Por ejemplo, un ácido graso con 20 átomos de carbono y 5 dobles enlaces, estando el primero en el tercer átomo de carbono contando desde el extremo metilo, se nombra 20:5n-3. Otra forma de nombrar a los ácidos grasos insaturados es haciendo referencia a su estructura química completa. Este sistema de nomenclatura contempla la posición del primer doble enlace contando desde el extremo carboxilo terminal del ácido graso. En este caso, las insaturaciones se identifican con la letra "delta (Δ)", de manera que un ácido graso con 20 átomos de carbono y 5 dobles enlaces en las posiciones 5, 8, 11, 14 y 17 se designa $20{:}5^{\Delta\ (5,8,11,14,17)}$ (**Figura 1.15**). Para poder determinar la posición de los dobles enlaces en la estructura de los ácidos grasos insaturados hay que tener en cuenta que, entre dos átomos de carbono insaturados siempre aparece un átomo de carbono carente de insaturación (**Figura 1.15**). Así, sabiendo la posición del primer doble enlace, podemos determinar la posición de los restantes. Existen excepciones, como es el caso de los ácidos grasos no interrumpidos por metilenos (en inglés *non-methylene-interrupted fatty acids*; NMI-FA), cuyas insaturaciones están separadas

por más de un grupo metileno (**Figura 1.16**). Aunque su identificación es bastante inusual, su presencia ha sido confirmada en algas e invertebrados marinos (Barnathan, 2009).

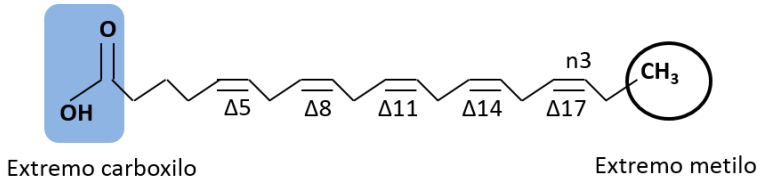


Figura 1.15. Ácido eicosapentaenoico (EPA), 20:5n-3/20:5$^{\Delta (5,8,11,14,17)}$.

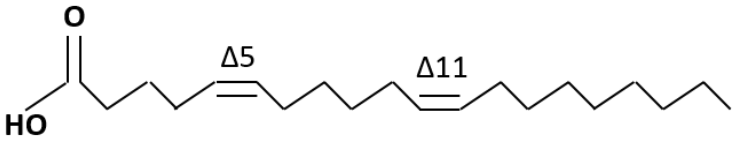


Figura 1.16. Estructura del *non-methylene-interrupted fatty acid* (NMI-FA) 20:2$^{\Delta (5,11)}$.

Aquellos ácidos grasos que presentan entre 20 y 24 átomos de carbono (C_{20-24}) en su estructura se denominan ácidos grasos de cadena larga (en inglés *long-chain fatty acids*; LC-FA) (**Figura 1.17 A**), diferenciándose, según su número de insaturaciones, en ácidos grasos saturados de cadena larga (en inglés *long-chain saturated fatty acids*; LC-SFA) y en ácidos grasos poliinsaturados de cadena larga (en inglés *long-chain polyunsaturated fatty acids*; LC-PUFA). Aquellos ácidos

grasos que presentan más de 24 átomos de carbono estructurales (>C_{24}) se denominan ácidos grasos de cadena muy larga (en inglés *very long-chain fatty acids*; VLC-FA) (**Figura 1.17 B**), diferenciándose a su vez, en ácidos grasos saturados de cadena muy larga (en inglés *very long-chain saturated fatty acids*; VLC-SFA) y en ácidos grasos poliinsaturados de cadena muy larga (en inglés *very long-chain polyunsaturated fatty acids*; VLC-PUFA).

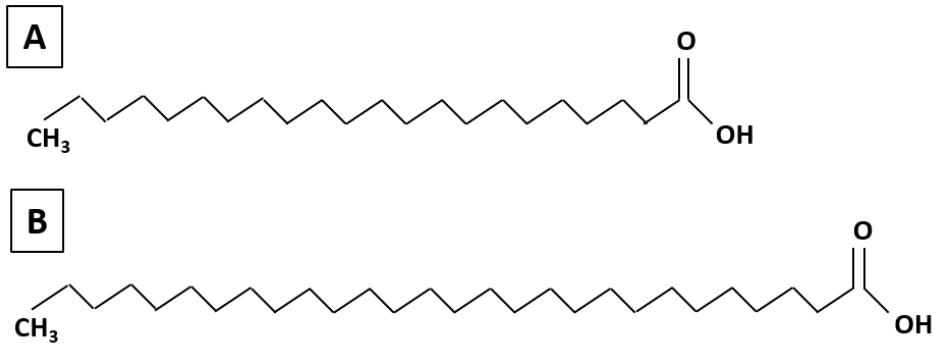


Figura 1.17. Ácido graso saturado de cadena larga (**A**) y ácido graso saturado de cadena muy larga (**B**).

1.6. Ácidos grasos de cadena larga (LC-FA) y muy larga (VLC-FA): metabolismo biosintético

Los ácidos grasos actúan como componentes básicos de diversas clases de lípidos presentes en las membranas celulares, siendo esenciales para la viabilidad celular (a excepción de *Archaea*). Su biosíntesis tiene lugar a nivel microsomal y/o

mitocondrial, a través de sucesivas elongaciones e insaturaciones, catalizadas por diferentes enzimas elongasas (en inglés *elongation of very long-chain fatty acid proteins*; Elovl) y desaturasas (en inglés *fatty acyl desaturases*; Fads). Estas enzimas actúan introduciendo átomos de carbono, en el caso de las Elovl, y dobles enlaces, en el caso de las Fads, en la cadena hidrocarbonada de los ácidos grasos más simples, que actúan como piedra angular en la biosíntesis de ácidos grasos de cadena larga (López-Lara, 2019).

Las enzimas Fads están presentes en multitud de organismos. Estas presentan una notable diversidad funcional. Así, en función de la posición de la cadena hidrocarbonada del ácido graso donde la Fads introduzca el doble enlace partiendo desde el extremo carboxilo terminal, se denomina su actividad desaturasa, pudiendo ser delta-4 (Δ4), si introduce una insaturación entre el carbono 4 y 5, delta-5 (Δ5), si introduce una insaturación en el carbono 5 y 6, delta-6 (Δ6), si introduce una insaturación entre el carbono 6 y 7, y así sucesivamente.

Los vertebrados carecen de enzimas Fads con actividad desaturasa delta-12 (Δ12) y delta-15 (Δ15), por lo que no pueden sintetizar PUFA de 18 carbonos *de novo*, que deben ser incorporados a través de la dieta (Monroig *et al*., 2018). Estas enzimas, presentes en los vegetales, protistas heterótrofos, bacterias y algunos metazoos invertebrados (Kabeya *et al*., 2018a), catalizan la formación secuencial del ácido linoleico (LA; 18:2n-6) y del ácido α-linolénico (ALA; 18:3n-3) a partir del

ácido oleico (18:1n-9). Ambos PUFA actúan como precursores en la síntesis endógena de LC-PUFA de vital importancia para el reino animal, como son el ácido araquidónico (ARA, 20:4n-6), el ácido eicosapentaenoico (EPA; 20:5n-3) y el ácido docosahexaenoico (DHA; 22:6n-3). Estas transformaciones se llevan a cabo a través de sucesivos ciclos de elongación/insaturación realizados a lo largo de la ruta de biosíntesis de los LC-PUFA (**Figura 1.18**).

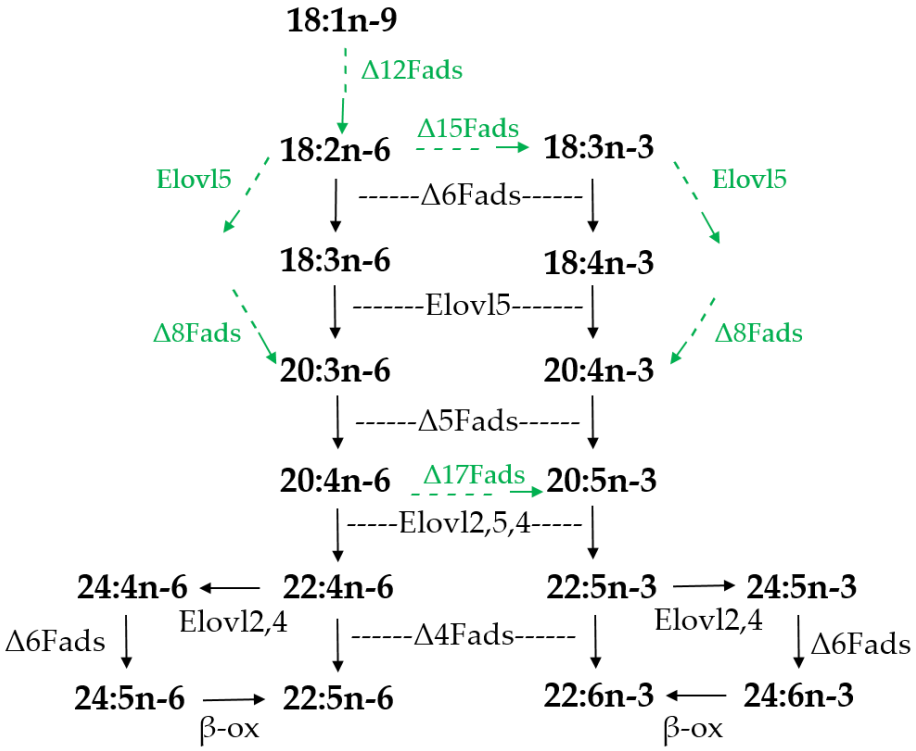


Figura 1.18. Ruta de biosíntesis de ácidos grasos poliinsaturados de cadena larga en vertebrados. Las flechas negras continuas indican vías confirmadas en teleósteos, mientras que las flechas verdes discontinuas indican vías confirmadas en otros organismos, aunque no en teleósteos.

A diferencia de los PUFA de 18 carbonos, es decir el LA y el ALA, los LC-PUFA, como el ARA, el EPA y el DHA, desempeñan un papel muy importante durante la reproducción, el desarrollo ontogénico, el crecimiento larvario, así como en procesos de reparación tisular en peces (Houston *et al*., 2017; Jobling *et al*., 2016). La biosíntesis de LC-PUFA a partir de LA y ALA está condicionada por el repertorio genético de desaturasas (*fads*) y elongasas (*elovl*), así como la especificidad de sustrato de las enzimas que codifican, asociadas a las diferentes especies de peces (Monroig *et al*., 2018).

Todas las enzimas desaturasas conocidas en teleósteos, a excepción de lo descrito en la anguila japonesa (*Anguilla japonica*), que presenta una Fads1 con actividad desaturasa Δ5 (Kabeya *et al*., 2018b; Lopes-Marques *et al*., 2018), son consideradas ortólogos de Fads2 (Castro *et al*., 2012). En peces, este grupo de proteínas enzimáticas, altamente conservadas, puede presentar actividad desaturasa Δ4, Δ5, Δ6 y/o Δ8 en función de la especie (Castro *et al*., 2016; Monroig *et al*., 2018). En el caso de la dorada (*S. aurata*) y el lenguado senegalés (*S. senegalensis*), su maquinaria de desaturación enzimática para la biosíntesis de DHA a partir de sus precursores difiere sustancialmente. La dorada posee una enzima Fads2 con actividad desaturasa Δ6, que presenta capacidad para bioconvertir precursores de 18 carbonos, como LA y ALA, en PUFA (Seiliez *et al*., 2003; Zheng *et al*., 2004). La biosíntesis de DHA vía "desaturasa Δ6" o "vía de Sprecher" requiere de dos

elongaciones adicionales, una segunda desaturación, y un acortamiento peroxisomal de la cadena hidrocarbonada (Sprecher, 2000) (**Figura 1.19**). Sin embargo, el lenguado senegalés posee una enzima Fads2 con actividad desaturasa Δ4 (Morais *et al*., 2012). La biosíntesis de DHA a partir de EPA vía "desaturasa Δ4", a diferencia de la vía "desaturasa Δ6", es más directa y eficiente, pues únicamente requiere una elongación previa, transformando el EPA en ácido docosapentaenoico (DPA, 22:5n-3), que posteriormente es desaturado en su posición Δ4 para generar DHA (Li *et al*., 2010) (**Figura 1.19**).

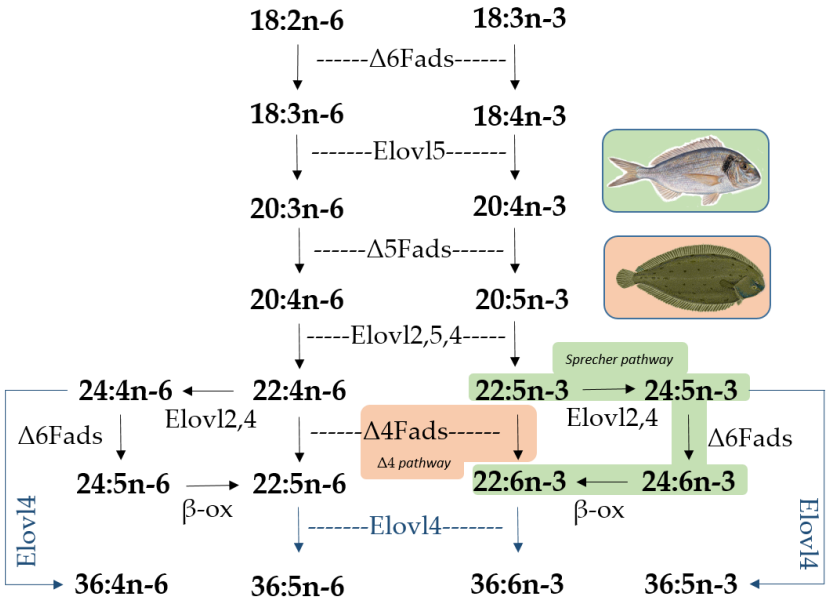


Figura 1.19. Ruta de biosíntesis de los ácidos grasos poliinsaturados de cadena larga (LC-PUFA; flechas negras) y de cadena muy larga (VLC-PUFA; flechas azules) en dorada (*Sparus aurata*) y lenguado (*Solea senegalensis*). La biosíntesis del ácido docosahexaenoico (DHA; 22:6n-3) a partir del ácido docosapentaenoico (DPA; 22:5n-3) aparece resaltada, diferenciándose dos rutas: la vía desaturasa Δ6 o "Sprecher pathway" llevada a cabo por la enzima Fads2 en *S. aurata* (verde) y la vía desaturasa Δ4 o "Δ4 pathway" llevada a cabo por la enzima Fads2 en *S. senegalensis* (rosa).

Estas diferencias específicas en la dotación de enzimas desaturasas pueden tener importantes repercusiones a nivel práctico, especialmente durante las primeras fases del desarrollo de los teleósteos marinos cultivados. En acuicultura, invertebrados acuáticos como los rotíferos (*Brachionus* sp.) y *Artemia* sp. son ampliamente utilizados como alimento vivo durante las fases larvarias de los peces cultivados. Estos organismos zooplanctónicos tienen una composición subóptima de LC-PUFA n-3 para el cultivo de la mayoría de teleósteos marinos, incluida la dorada (Sargent *et al*., 1999). Este déficit nutricional, unido a la mayor complejidad y la teóricamente insuficiente tasa de producción endógena de DHA, llevada a cabo en la dorada por la Fads2 a través de la vía de Sprecher (desaturasa Δ6), hacen que sea necesario recurrir al uso de enriquecedores comerciales que mejoren la calidad nutritiva de las presas vivas. Sin embargo, este enriquecimiento no es necesario en el cultivo del lenguado senegalés, que ve reducida las exigencias nutricionales de DHA debido a la mayor eficiencia biosintética de su Fads2 con actividad desaturasa Δ4. Esto hace posible el uso de especies auxiliares no enriquecidas como alimento durante la primera fase de alimentación exógena sin que afecte significativamente al desarrollo, al crecimiento y a la supervivencia larvaria de esta especie (Morais *et al*., 2004; Villalta *et al*., 2005).

De entre las diferentes elongasas descritas en vertebrados (Elovl1-7), además de una nueva Elovl8, cuya posible intervención se ha sugerido recientemente (Li *et*

al., 2020), sólo las enzimas Elovl2, Elovl4 y Elovl5 participan en la elongación de los PUFA (Castro *et al*., 2016; Jakobsson *et al*., 2006). Estas enzimas catalizan la primera reacción de condensación llevada a cabo durante el proceso de elongación, que resulta en la adición de dos átomos carbono a la cadena del ácido graso preexistente (Jakobsson *et al*., 2006). Sin embargo, la mayoría de los peces marinos, debido a la pérdida evolutiva de la Elovl2, solo poseen una isoforma de la Elovl5 y dos de la Elovl4, denominadas Elovl4a y Elovl4b (Castro *et al*., 2016; Monroig *et al*., 2018). En peces, la enzima Elovl5 participa en la biosíntesis de LC-PUFA a través de la elongación de sustratos PUFA de 18-20 carbonos, presentando una eficiencia de conversión variable en función de la especie (Monroig *et al*., 2018). Las enzimas Elovl4 están involucradas en la biosíntesis de los VLC-FA, es decir los VLC-PUFA y los VLC-SFA (**Figura 1.20**), mediante la elongación de una amplia gama de sustratos de ácidos grasos, pudiendo éstos diferir entre las diferentes especies de teleósteos (Betancor *et al*., 2020; Carmona-Antoñanzas *et al*., 2011; Ferraz *et al*., 2020; Jin *et al*., 2017; Kabeya *et al*., 2015; Monroig *et al*., 2010, 2011; Oboh *et al*., 2017).

Las Elovl4 también desempeñan un papel importante en la vía de biosíntesis de LC-PUFA. Estas enzimas muestran una funcionalidad similar a la de la Elovl5 para elongar activamente PUFA de 18-20 carbonos hasta DHA (Ferraz *et al*., 2020; Xie *et al*., 2016; Yan *et al*., 2018) y compensan, en parte, la falta de la Elovl2 en muchas

especies de teleósteos marinos (Betancor *et al*., 2020; Monroig *et al*., 2011, 2018; Sun *et al*., 2020). Debido a la doble funcionalidad descrita anteriormente, y al papel clave que juegan sus productos de biosíntesis, es decir los VLC-FA, en el desarrollo y la correcta funcionalidad cerebral y retiniana, el gen codificante para la enzima Elovl4 (*elovl4*) se considera un gen crucial fuertemente conectado a la función neuronal de los vertebrados, incluidos los peces (Agbaga *et al*., 2010; Deák *et al*., 2019; Monroig *et al*., 2010, 2011; Sherry *et al*., 2017).

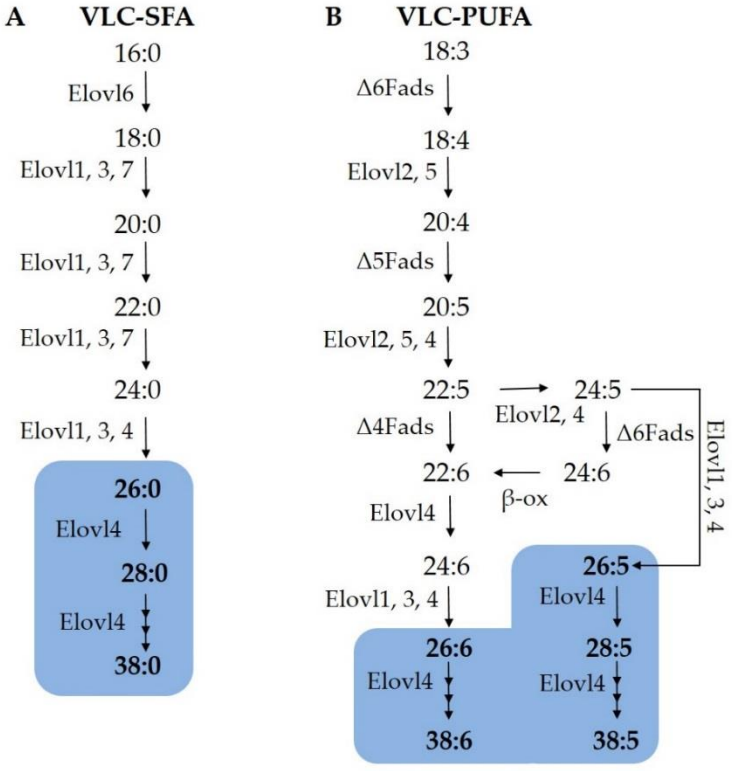


Figura 1.20. Rutas de elongación de los VLC-SFA (A) y de los VLC-PUFA (B). En negrita, y rodeados por un recuadro azul se destacan los ácidos grasos >C_{24}.

Distintas evidencias otorgan a los VLC-FA importantes funciones en diversos procesos fisiológicos, como la visión, la función cerebral, la permeabilidad de la piel y la reproducción de los mamíferos (Agbaga *et al*., 2010; Aldahmesh *et al*., 2011; Deák *et al*., 2019; Furland *et al*., 2007; Gage *et al*., 2003; Poulos, 1995). Sin embargo, a pesar de que varios estudios llevados a cabo en diferentes especies de peces con importancia comercial han enfatizado la importancia de los VLC-FA en el sector acuícola (Betancor *et al*., 2020; Carmona-Antoñanzas *et al*., 2011; Ferraz *et al*., 2020; Jin *et al*., 2017b; Monroig *et al*., 2010, 2011; Oboh *et al*., 2017; Zhao *et al*., 2019), existen numerosas incógnitas sobre la presencia, concentración, funcionalidad, distribución tisular y regulación nutricional de la biosíntesis de estos compuestos en las diferentes especies de teleósteos cultivadas. Las razones para ello son su baja concentración tisular, su complejidad analítica y la falta de patrones de referencia comercialmente disponibles que ayuden a su correcta identificación (Agbaga *et al*., 2010; Garlito *et al*., 2019). Por ello, la mayoría de los estudios que se han llevado a cabo en organismos acuáticos, se restringen a la caracterización funcional de las enzimas Elovl4 encargadas de su biosíntesis (Betancor *et al*., 2020; Carmona-Antoñanzas *et al*., 2011; Ferraz *et al*., 2020; Jin *et al*., 2017; Kabeya *et al*., 2015; Monroig *et al*., 2010; Oboh *et al*., 2017).

De manera generalizada, se ha asociado la capacidad de producir VLC-FA a las dos isoformas de Elovl4, es decir Elovl4a y Elovl4b. En consecuencia, la capacidad biosintética de ambas enzimas, así como su adecuada transcripción-traducción a nivel tisular, son claves para determinar los hipotéticos requerimientos de VLC-FA asociados a cada especie (Deák *et al*., 2019; Ferraz *et al*., 2020; Jin *et al*., 2017; Monroig *et al*., 2010; Oboh *et al*., 2017). Por lo general, Elovl4a se ha relacionado con la biosíntesis de VLC-SFA, mientras que Elovl4b ha sido asociada con la biosíntesis de VLC-SFA y VLC-PUFA (Deák *et al*., 2019). Los VLC-SFA y los VLC-PUFA, debido a la longitud de su cadena hidrocarbonada, confieren propiedades únicas a los complejos lipídicos y, por ende, a las membranas celulares en las que éstos son incorporados. Los VLC-FA se hallan presentes a lo largo de todo el sistema nervioso central, estando fuertemente ligados a la correcta funcionalidad neuronal (Deák *et al*., 2019). Además, los VLC-PUFA se encuentran mayoritariamente asociados a las moléculas de fosfatidilcolina presentes en la retina (**Figura 1.21**), ejerciendo una función clave en la protección de las células fotorreceptoras (Agbaga *et al*., 2010; Deák *et al*., 2019; McMahon *et al*., 2007). A su vez, los VLC-SFA son incorporados en el cerebro y en la piel principalmente en forma de esfingolípidos y ceramidas (**Figura 1.22**), respectivamente (Deák *et al*., 2019). Los VLC-PUFA, debido a su longitud y a los múltiples metilenos (CH_2) interrumpidos por dobles enlaces *cis*, que favorecen el desempaquetamiento de los

fosfolípidos en las membranas celulares, aumentan su fluidez, pudiendo incluso modificar su curvatura (Antonny *et al*., 2015; Lauwers *et al*., 2016). En contraste, la gran longitud de la cadena lineal de los VLC-SFA les confiere una alta temperatura de fusión, aumentando la rigidez de las membranas celulares debido a las fuertes interacciones de Van der Waals establecidas entre las cadenas adyacentes (Hopiavuori *et al*., 2018).

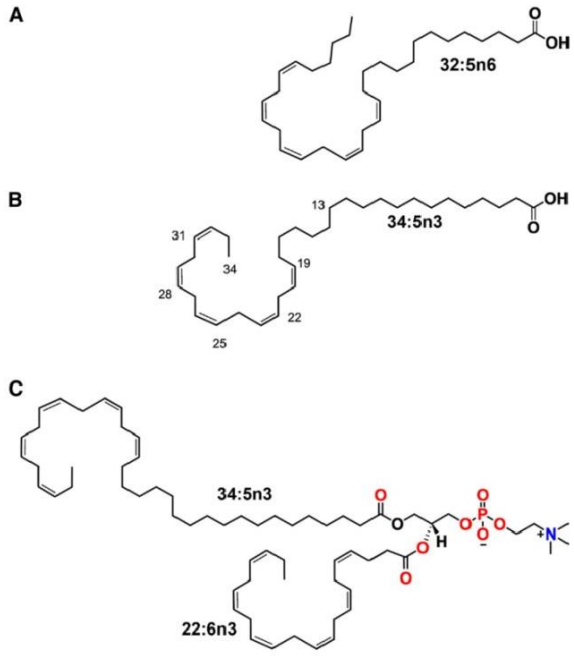


Figura 1.21. Estructura de los VLC-PUFA. Forma libre del VLC-PUFA 32:5n-6 (**A**) y 34:5n-3 (**B**). VLC-PUFA esterificado en la posición *sn-1* del esqueleto de glicerol, formando parte de la estructura de un fosfolípido (**C**). Imagen extraída de Agbaga *et al*., 2010.

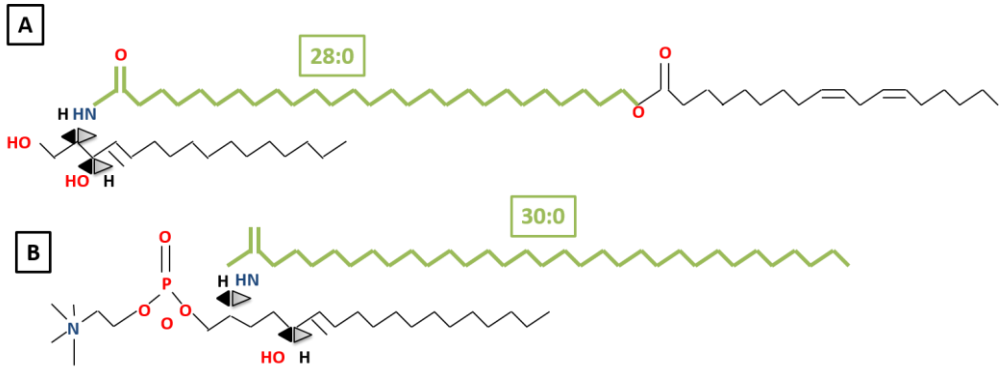


Figura 1.22. VLC-SFA incorporados en ceramida (**A**) y en esfingolípido (**B**).

A pesar de la importancia que los VLC-PUFA tienen para el correcto desarrollo y funcionalidad celular (Deák *et al*., 2019), se desconoce el efecto que la restricción en el suministro dietario de sus precursores, los LC-PUFA, puede ejercer en la biosíntesis de estos ácidos grasos "superesenciales" en peces cultivados. En la producción acuícola, la restricción dietaria de los LC-PUFA se está convirtiendo en una tendencia al alza debido a la escasa disponibilidad de ingredientes marinos, ricos en LC-PUFA, como la harina y el aceite de pescado (Shepherd *et al*., 2017). Por ello, es importante investigar los mecanismos moleculares involucrados en la síntesis endógena de los VLC-PUFA, especialmente durante las primeras etapas del desarrollo de los peces.

Teniendo en cuenta que la expresión de los genes *fads* y *elovl* puede ser nutricionalmente regulada a través del contenido en ácidos grasos presente en la

dieta (Izquierdo *et al*., 2008; Kuah *et al*., 2015; Li *et al*., 2016; Li *et al*., 2017; Morais *et al*., 2012), es importante llevar a cabo estudios centrados en la posible regulación nutricional de los genes *elovl4* como recurso para estimular la biosíntesis de los VLC-PUFA en peces cultivados. Dichos trabajos deben considerar la capacidad endógena que cada especie tiene para producir los VLC-PUFA, que depende de la dotación de los genes *elovl4* y de las funciones de sus correspondientes enzimas codificadas (Monroig *et al*., 2018). Además, se debe atender a las características nutricionales de los diferentes tipos de dietas empleadas durante las primeras etapas del desarrollo de los telósteos, así como las diferentes rutas biosínteticas que operan, a nivel de especie, en el proceso de biosíntesis de los LC-PUFA. Así, esta tesis pretende contribuir al esclarecimiento de la regulación de los patrones de expresión de los genes involucrados en la biosintésis de los VLC-PUFA y su análisis desde un punto de vista comparativo interespecífico y ontogénico, tal y como se decribe en el siguiente capítulo.

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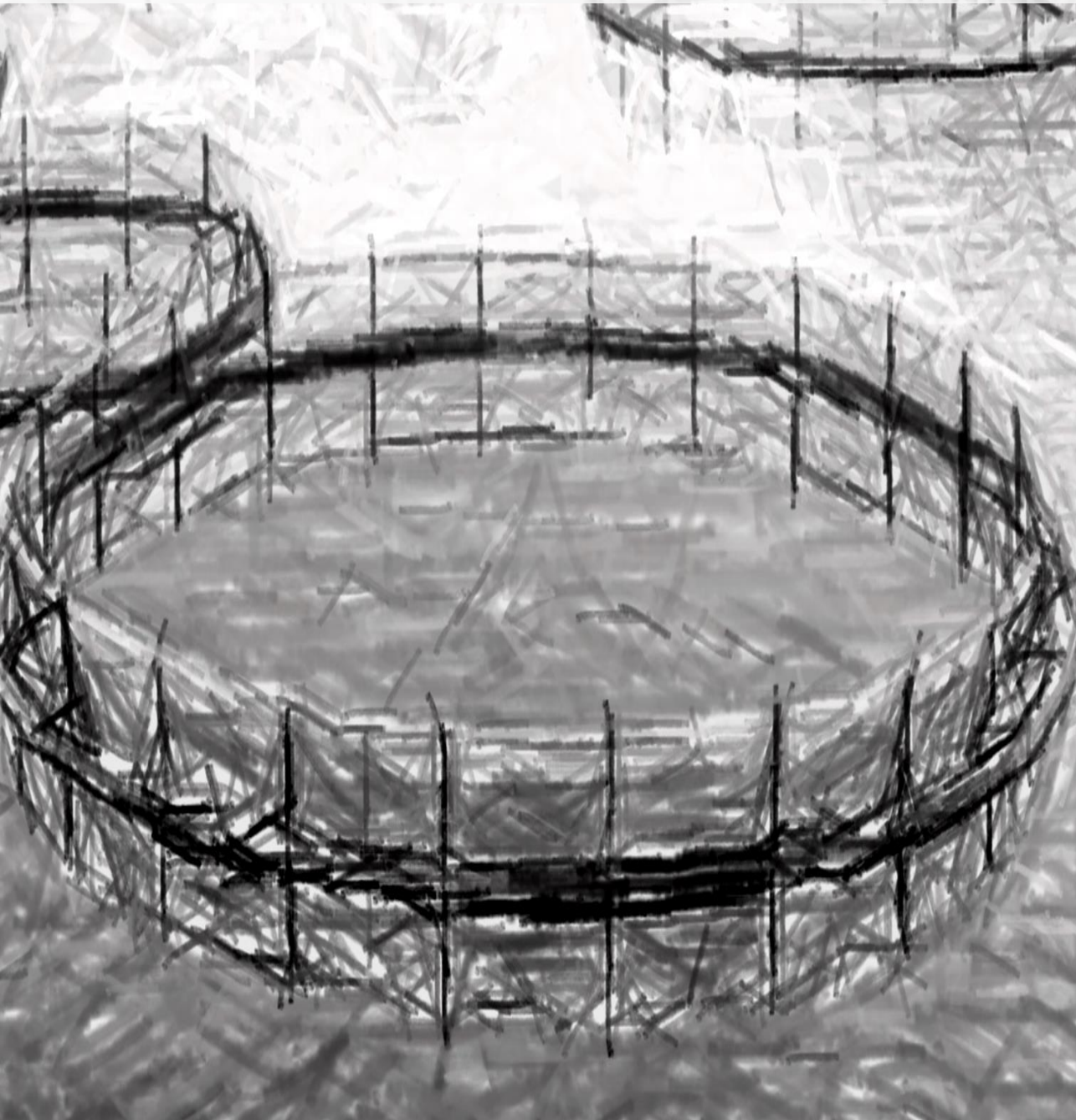
CAPÍTULO 2:

Objetivos y presentación de los trabajos



2.1. Objetivo general

El objetivo del trabajo de investigación llevado a cabo en la presente Tesis Doctoral, ha sido ampliar el conocimiento sobre la biosíntesis de los VLC-FA en dos especies de gran interés para la acuicultura mediterránea, como son la dorada y el lenguado senegalés. Para ello, se ha caracterizado el repertorio de genes *elovl4* y estudiado la funcionalidad de las enzimas que codifican (Elovl4). Asimismo, con el fin de determinar el mecanismo de síntesis y el potencial rango de presencia a nivel tisular de los VLC-FA, se ha estudiado el patrón de expresión espacio-temporal de *elovl4a* y *elovl4b* en las dos especies. Además, se ha investigado la influencia de la dieta sobre los patrones de expresión de distintos genes involucrados en la formación endógena de los VLC-PUFA (*elovl4a* y *elovl4b*), así como la de sus precursores biosintéticos, es decir los LC-PUFA (*fads2* y *elovl5*), a lo largo del desarrollo larvario y poslarvario de ambas especies.

2.2. Objetivos específicos

1. Caracterización molecular y funcional, respectivamente, de los parálogos de *elovl4* (*elovl4a* y *elovl4b*) y de las proteínas que codifican (Elovl4a y Elovl4b) en la dorada (*Sparus aurata*) y en el lenguado senegalés (*Solea senegalensis*).

2. Determinación de los patrones de expresión tisular de *elovl4a* y *elovl4b* en la dorada y el lenguado senegalés.

3. Determinación de los patrones de expresión temporal de los genes codificantes para la desaturasa Fads2, y las elongasas Elovl5, Elovl4a y Elovl4b, durante el desarrollo temprano de larvas de dorada y de lenguado senegalés.

4. Estudio de la regulación nutricional de los genes *fads2*, *elovl5*, *elovl4a* y *elovl4b* por medio de dietas con diferente contenido en LC-PUFA durante las fases tempranas del desarrollo en la dorada y el lenguado senegalés.

4.1. Regulación nutricional larvaria de *fads2*, *elovl5*, *elovl4a* y *elovl4b* por parte de dietas consistentes en presas vivas, rotíferos (*Brachionus* sp.) y *Artemia* sp., enriquecidas y no enriquecidas.

4.2. Regulación nutricional poslarvaria de los genes involucrados en la biosíntesis de LC- y VLC-PUFA mediante el empleo de microdietas inertes formuladas con un gradiente de LC-PUFA.

2.3. Presentación de los trabajos y justificación de la unidad temática

Teniendo en cuenta las características estructurales únicas de los VLC-FA, su esencialidad y funcionalidad como componente vital de las membranas celulares presentes en estructuras fisiológicas transcendentales, como son el cerebro y la retina de organismos vertebrados (Deák *et al*., 2019), y dado que por su dificultad analítica y escasa presencia aún no han sido suficientemente investigados, el estudio de estos

ácidos grasos en el contexto de la acuicultura es de gran importancia para valorar los requerimientos específicos y la capacidad endógena de biosíntesis que las diferentes especies de peces marinos cultivados presentan, especialmente durante las fases tempranas del desarrollo. Por ello, los principales propósitos de este trabajo de investigación son el estudio de la caracterización molecular y el de los patrones de expresión espacio-temporal de los genes involucrados en la biosíntesis de los VLC-PUFA (*elovl4a* y *elovl4b*) y en la de sus precursores, los LC-PUFA (*fads2* y *elovl5*), durante el desarrollo larvario de dos de las principales especies cultivadas en el Mediterráneo como son la dorada y el lenguado senegalés. La elección de estas especies se basa en las diferentes rutas enzimáticas que operan en el metabolismo biosintético de los LC-PUFA. Así, durante el desarrollo de este trabajo de investigación, ambas especies son consideradas como modelos comparativos de la síntesis endógena de DHA a través de la "vía desaturasa Δ6 o vía de Sprecher", en el caso de la dorada, y de la "vía desaturasa Δ4", en el caso del lenguado senegalés. Además, se pretende estudiar la caracterización funcional de las proteínas Elovl4a y Elovl4b que controlan la ruta anabólica de los VLC-PUFA, codificadas por los genes anteriormente mencionados.

Paralelamente, con el fin de comprender los impactos que las formulaciones actuales de alimentos acuícolas con niveles reducidos de LC-PUFA puedan tener, y apoyados en la ya demostrada influencia que el contenido dietario en ácidos grasos

ejerce sobre la expresión de los genes *fads* y *elovl* (Monroig *et al*., 2018), se propone la regulación nutricional de los genes *fads2*, *elovl5*, *elovl4a* y *elovl4b*, como una posible línea de investigación para mejorar la producción endógena de los VLC-PUFA y los LC-PUFA. Estos últimos actúan, además, como precursores biosintéticos de los correspondientes VLC-PUFA. En consecuencia, es fundamental comprender la capacidad que tiene una especie determinada para la producción endógena de estos nutrientes, así como conocer el efecto que la entrada de nutrientes a través de la dieta puede ejercer sobre la expresión de los genes en estudio. Por ello, esta investigación va dirigida a una mejor comprensión de los mecanismos de regulación nutricional que influyen sobre la expresión de los genes *fads2*, *elovl5*, *elovl4a* y *elovl4b*, especialmente durante las primeras etapas del ciclo de vida de los peces cultivados. Es durante esta fase cuando las larvas y las poslarvas necesitan un mayor suplemento dietario de LC-PUFA, que les permita afrontar los grandes cambios fisiológicos que experimentan durante su rápido desarrollo y crecimiento (Hamre *et al*., 2013, Monroig *et al*., 2018).

Dentro de estos planteamientos se justifica la unidad temática de los trabajos de investigación ya publicados, que componen esta Tesis Doctoral. De esta manera, se incide en la necesidad de mejorar la calidad nutritiva del alimento que se ofrece durante las primeras fases del desarrollo los peces marinos cultivados, concretamente

en relación a su contenido en ácidos grasos de cadena larga. Estos trabajos se corresponden con los capítulos del 3 al 5, que son presentados a continuación.

Capítulo 3: Molecular and functional characterization of *elovl4* genes in *Sparus aurata* and *Solea senegalensis* pointing to a critical role in very long-chain (>C_{24}) fatty acid synthesis during early neural development of fish.

La funcionalidad de los productos de síntesis de la Elovl4 ha sido ampliamente descrita en vertebrados superiores, principalmente en mamíferos, donde los VLC-SFA y los VLC-PUFA desempeñan un papel vital en los procesos de neuroprotección y neurotransmisión (Deák *et al*., 2019). Esto es especialmente importante durante el desarrollo temprano de los vertebrados (Sherry *et al*., 2017), ya que es durante el proceso de formación del sistema nervioso, cuando una correcta biosíntesis y acumulación de estos compuestos es vital para garantizar su correcto desarrollo y actividad neural. Basándonos en ello, el objetivo del estudio fue caracterizar molecularmente los parálogos de *elovl4* en la dorada (*S. aurata*) y el lenguado senegalés (*S. senegalensis*), mediante la clonación molecular de secuencias de ADNc de *elovl4.* Además, se determinó el patrón de expresión tisular de los genes *elovl4a* y *elovl4b* mediante el empleo de técnicas PCR. Asimismo, se caracterizaron funcionalmente las enzimas codificadas por lo genes mencionados, Elovl4a y Elovl4b, mediante la expresión de sus proteínas heterólogas en levadura. De esta

manera, se pretendió investigar la potencial importancia que los VLC-FA tienen para el correcto desarrollo de las especies objeto de estudio, especialmente durante su desarrollo temprano.

Capítulo 4: Expression of genes related to long-chain (C_{20-24}) and very long-chain (>C_{24}) fatty acids biosynthesis in gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*): Investigating early ontogeny and nutritional regulation.

Partiendo de la importancia que los VLC-FA muestran para el correcto desarrollo y funcionalidad de estructuras cognitivas vitales, como son el cerebro y la retina, especialmente durante las primeras etapas del desarrollo de los vertebrados (Deák *et al*., 2019; Sherry *et al*., 2017), el primer objetivo de este ensayo fue determinar los patrones de expresión temporal de los genes involucrados en la biosíntesis de los VLC-PUFA, *elovl4a* y *elovl4b*, y de sus precursores, los LC-PUFA, *fads2* y *elovl5*, durante la ontogenia temprana de la dorada y el lenguado senegalés. Para ello, se diseñó un primer experimento donde se muestrearon diariamente huevos (pre-eclosión) y larvas de *S. aurata* y *S. senegalensis* con una edad de 0 a 7 días. El segundo objetivo fue estudiar la regulación nutricional de dichos genes durante la fase larvaria de ambas especies, cuándo éstas comienzan a alimentarse de manera exógena. Para ello, larvas de ambas especies fueron

alimentadas con presas vivas mediante el uso de especies auxiliares en acuicultura, enriquecidas y no enriquecidas en LC-PUFA, como el rotífero *Brachionus plicatilis* y el crustáceo braquiópodo anostráceo *Artemia franciscana*. Posteriormente, para ambos ensayos, se analizó el patrón de expresión de los genes mencionados mediante la técnica q-PCR

Capítulo 5: Nutritional regulation of genes responsible of long-chain (C_{20-24}) and very long-chain (>C_{24}) fatty acids biosynthesis in post-larvae of gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*).

Dado el actual contexto de formulación de dietas destinadas a la acuicultura, caracterizado por la tendencia a substituir/reducir productos de origen animal por productos de origen vegetal (Shepherd *et al*., 2017; Turchini *et al*., 2011), lo que implica una reducción del aporte dietario de LC-PUFA n-3 (Tocher, 2015), el objetivo de este ensayo fue investigar la regulación nutricional de los genes involucrados en la biosíntesis de los LC- y VLC-PUFA, así como determinar el efecto que el contenido dietario en LC-PUFA puede ejercer sobre el crecimiento y la composición en ácidos grasos durante el desarrollo poslarvario de *S. aurata* y *S. senegalensis*. Para tal fin, poslarvas de ambas especies fueron alimentadas con tres microdietas inertes formuladas con diferentes niveles de combinación de aceite de pescado y aceite de soja, resultando en un contenido variable en LC-PUFA n-3,

principalmente en EPA y DHA. Posteriormente, se analizó el efecto dietario sobre los parámetros de crecimiento, el perfil tisular de ácidos grasos y la expresión de los genes *fads2*, *elovl5*, *elovl4a* y *elovl4b*. Así, los resultados obtenidos pueden ayudar a un mejor conocimiento de la biosíntesis endógena de los VLC-PUFA y sus precursores, los LC-PUFA, en teleósteos marinos cultivados.

Finalmente, el capítulo 6 incluye una discusión general, abordando de manera integral las cuestiones planteadas durante el desarrollo de la presente Tesis Doctoral.

*Nota: Los capítulos del 3 al 5 mantienen los requisitos establecidos por las revistas donde fueron publicados, aunque han sido editados para facilitar su lectura y mantener una uniformidad, así como para adaptarlos al formato de la presente Tesis Doctoral.

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CAPÍTULO 3:

Molecular and functional characterization of *elovl4* genes in *Sparus aurata* and *Solea senegalensis* pointing to a critical role in very long-chain (>C_{24}) fatty acid synthesis during early neural development of fish

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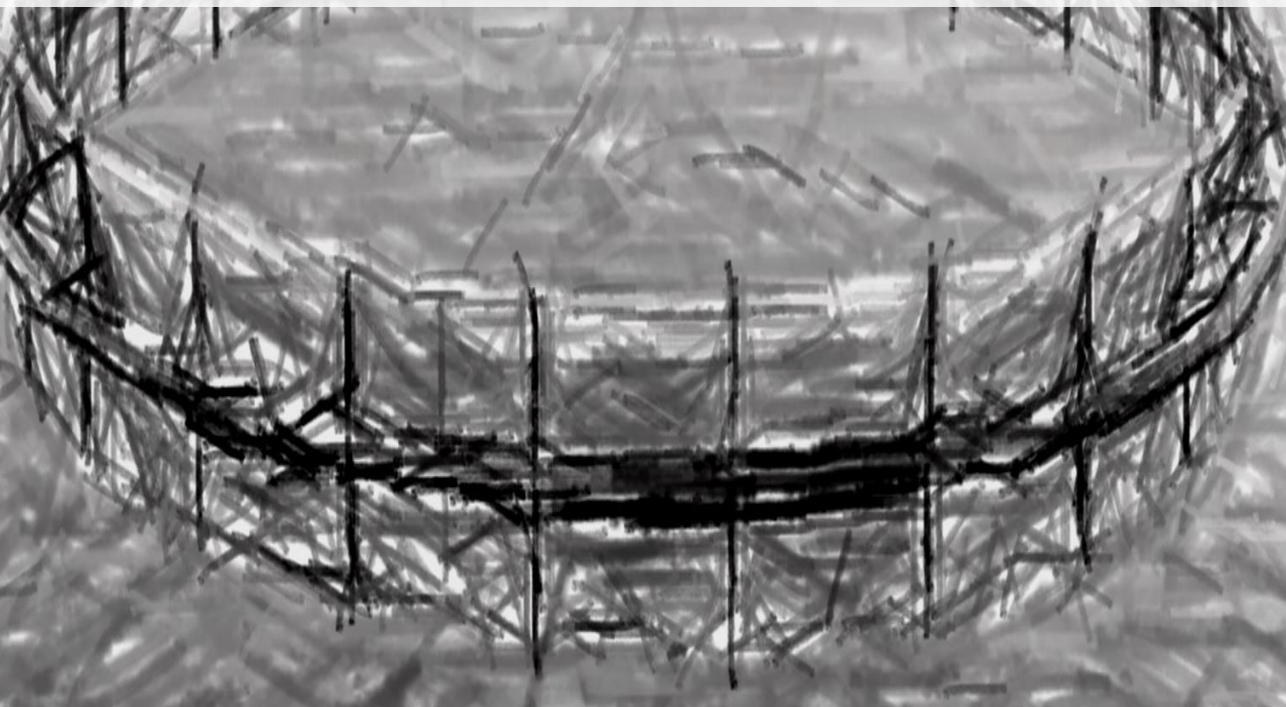

Abstract

Very long-chain fatty acids (VLC-FA) play critical roles in neural tissues during the early development of vertebrates. However, studies on VLC-FA in fish are scarce. The biosynthesis of VLC-FA is mediated by elongation of very long-chain fatty acid 4 (Elovl4) proteins and, consequently, the complement and activity of these enzymes determines the capacity that a given species has for satisfying its physiological demands, in particular for the correct development of neurophysiological functions. The present study aimed to characterize and localize the expression of *elovl4* genes from *Sparus aurata* and *Solea senegalensis*, as well as to determine the function of their encoded proteins. The results confirmed that both fish possess two distinct *elovl4* genes, named *elovl4a* and *elovl4b*. Functional assays demonstrated that both Elovl4 isoforms had the capability to elongate long-chain ($C_{20–24}$), both saturated (SFA) and polyunsaturated (PUFA), fatty acid precursors to VLC-FA. In spite of their overlapping activity, Elovl4a was more active in VLC-SFA elongation, while Elovl4b had a preponderant elongation activity towards n-3 PUFA substrates, particularly in *S. aurata*, being additionally the only isoform that is capable of elongating docosahexaenoic acid (DHA). A preferential expression of *elovl4* genes was measured in neural tissues, being *elovl4a* and *elovl4b* mRNAs mostly found in brain and eyes, respectively.

Keywords: Gilthead seabream; Senegalese sole; Very long-chain polyunsaturated fatty acid; Elovl4; Functional characterization; Tissue expression; Neural tissue development.

3.1. Introduction

Certain long-chain (C_{20-24}) polyunsaturated fatty acids (LC-PUFA), namely eicosapentaenoic acid (EPA; 20:5n-3), arachidonic acid (ARA; 20:4n-6) and docosahexaenoic acid (DHA; 22:6n-3), are regarded as physiologically essential for the correct development of vertebrates, including fish (Monroig *et al*., 2018). These compounds can be obtained through the diet or, alternatively, biosynthesized from C_{18} polyunsaturated fatty acids (PUFA), such as α-linolenic acid (18:3n-3) and linoleic acid (18:2n-6), via enzymatic reactions carried out by fatty acyl desaturases (Fads) and elongation of very long-chain fatty acid (Elovl) proteins (Castro *et al*., 2016; Monroig *et al*., 2018). Fads are enzymes that introduce double bonds (unsaturations) into PUFA substrates. On the other hand, Elovl are considered to be pivotal components of fatty acid (FA) synthetic pathways (Guillou *et al*., 2010; Jakobsson *et al*., 2006), being responsible for a condensation reaction, which results in the extension of the pre-existing FA chain with two new carbon atoms (Monroig *et al*., 2018). The Elovl protein family contains several members (Castro *et al*., 2016; Guillou *et al*., 2010; Jakobsson *et al*., 2006), of which only a few have been demonstrated to have PUFA as substrates. Of these, Elovl2, Elovl4 and Elovl5, have well-established roles in the biosynthesis of LC-PUFA in vertebrates (Castro *et al*., 2016; Guillou *et al*., 2010; Jakobsson *et al*., 2006), while a novel Elovl8 has been more recently suggested to be also involved in PUFA elongation (Li *et al*., 2020;

Oboh, 2018). While Elovl2 and Elovl5 are primarily involved in elongation steps within the LC-PUFA biosynthesis pathway, Elovl4 catalyzes the synthesis of very long-chain (>C_{24}) PUFA (VLC-PUFA), which can have up to 36 or 38 carbons (Agbaga *et al*., 2010; Monroig *et al*., 2018). Furthermore, Elovl4 is additionally responsible for the production of very long-chain saturated fatty acids (VLC-SFA) (Deák *et al*., 2019).

Virtually all teleosts possess at least two Elovl4 isoforms termed as "Elovl4a" and "Elovl4b" (Castro *et al*., 2016; Monroig *et al*., 2010). Gene expression data indicates that both *elovl4* paralogs have widespread tissue distribution, with *elovl4a* being highly expressed in brain (Monroig *et al*., 2010; Oboh *et al*., 2017) and *elovl4b* in eye (retina) and gonads (Monroig *et al*., 2010; Oboh *et al*., 2017). The functions of Elovl4a and Elovl4b seem to vary among species. For instance, in zebrafish (*Danio rerio*), Elovl4a showed the ability to elongate saturated FA (SFA) to produce VLC-SFA, while only Elovl4b was able to elongate PUFA substrates to produce VLC-PUFA (Monroig *et al*., 2010). However, studies performed on African catfish (*Clarias gariepinus*) (Oboh *et al*., 2017) and black seabream (*Acanthopagrus schlegelii*) (Jin *et al*., 2017) have demonstrated that both Elovl4a and Elovl4b have the ability to biosynthesize VLC-PUFA. These results suggest that the investigation of Elovl4 proteins in teleosts requires a species-specific approach.

The gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*) are two commercially important species in marine finfish aquaculture. A recent study highlighted a relationship between the expression of *elovl4* genes in both species and the formation of neural tissues during early life-cycle development (Torres *et al*., 2020a). Indeed, Elovl4 products, i.e., VLC-SFA and VLC-PUFA, play crucial roles during the early-development of vertebrates by guaranteeing the correct development and functionality of the rapidly forming nervous system, where these compounds accumulate (Deák *et al*., 2019; Torres *et al*., 2020a). From what is known in higher vertebrates, VLC-PUFA are generally incorporated into phosphatidylcholine in the photoreceptor cells that make up the retina (Agbaga *et al*., 2010), and are then bioconverted into elovanoids, which participate in photoreceptor protection (Bazan, 2018; Deák *et al*., 2019). On the other hand, VLC-SFA are mainly incorporated into sphingolipids in the brain (Oboh *et al*., 2017), taking part in the membrane fusion of synaptic vesicles that occur during the neurotransmission process in mammals (Hopiavuori *et al*., 2018, 2019). Finally, Elovl4, including teleost Elovl4, can also play a role in the biosynthesis of LC-PUFA, specifically DHA (Xie *et al*., 2016; Yan *et al*., 2018), which is the most abundant FA in brain and retinal cells (Mourente, 2003; Stoknes *et al*., 2004; Tocher and Harvie, 1988).

It is crucial to understand the capacity that a given species has for endogenous production of these essential nutrients due to the importance of very long-chain fatty

acids (VLC-FA) during early development. Such ability is itself dependent on the complement of *elovl4* genes and the functions of their corresponding encoded enzymes (Monroig *et al*., 2018). Having this in mind, the aim of the present study was to characterize, both molecularly and functionally, *elovl4* paralogs from *S. aurata* (*Sa*) and *S. senegalensis* (*Ss*). Previous studies investigating the functions of *fads*- and other *elovl*-like genes confirmed that both species operate different LC-PUFA biosynthesis mechanisms (Agaba *et al*., 2005; Morais *et al*., 2012; Seiliez *et al*., 2003; Zheng *et al*., 2004), especially with regard to the production of DHA. In particular, *Sa* operates the so-called "Sprecher pathway" (Seiliez *et al*., 2003; Sprecher, 2000), whereas *Ss* produces DHA via the more direct "Δ4 pathway" (Morais *et al*., 2012) (**Figure 3.1**). We will discuss our results in the context of the biosynthetic particularities of both species when considering that both the LC-PUFA and VLC-PUFA biosynthetic pathways are interdependent.

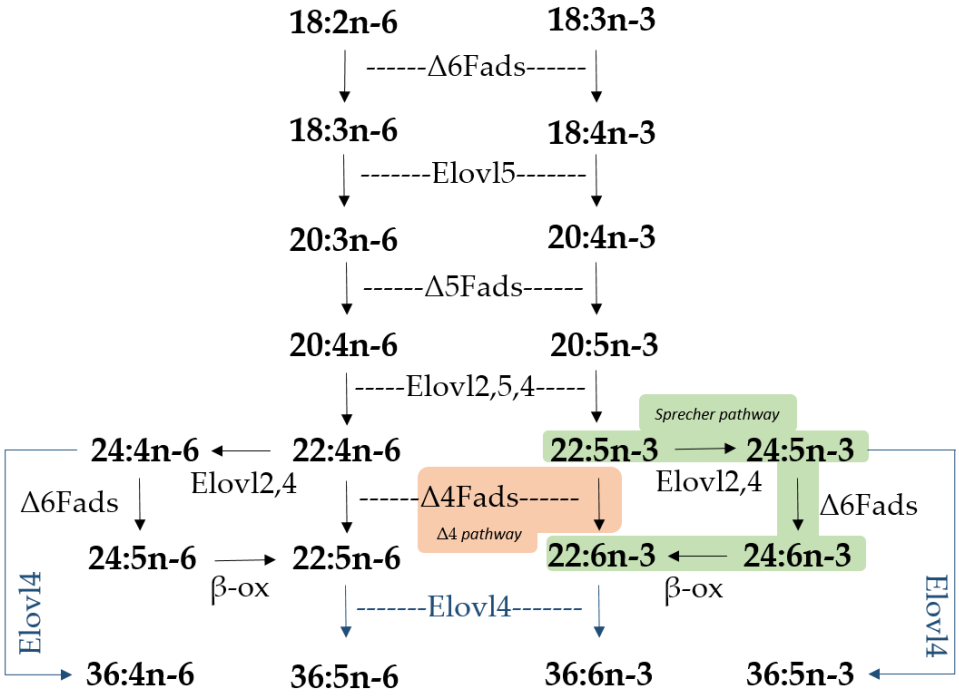

Figure 3.1. Biosynthetic pathways of long-chain (LC-PUFA; $C_{20–24}$) and very long-chain polyunsaturated fatty acids (VLC-PUFA; $>C_{24}$) in fish. Desaturation reactions are mediated by fatty acyl desaturases (Fads), whereas elongation reactions are catalyzed by elongation of very long-chain fatty acid (Elovl) proteins. Microsomal β-oxidation reactions are denoted as "β-ox". Two pathways for docosahexaenoic acid (DHA; 22:6n-3) biosynthesis from docosapentaenoic acid (DPA; 22:5n-3) are indicated, namely the Sprecher pathway (green background) and the Δ4 pathway (pink background). Elongation reactions leading to VLC-PUFA biosynthesis of up to C_{36} are indicated with blue arrows. Note the fish species studied herein (*Sparus aurata* and *Solea senegalensis*) lack *elovl2* in their genomes (Castro *et al*., 2016).

3.2. Materials and Methods

All of the experimental procedures were conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes, at the Instituto de Acuicultura de Torre de la Sal (IATS-CSIC). The Animal Welfare

and Bioethical Committee of IATS-CSIC approved all experimental conditions and sampling protocols under the code 015/2013 on 24 January 2014 according to Royal Decree RD53/2013.

3.2.1. Molecular cloning of elovl4 cDNA sequences

Total RNA was isolated from gilthead seabream and Senegalese sole brain mass and eye using Maxwell 16 LEV simplyRNA Tissue Kit (Promega Biotech Ibérica S.L., Madrid, Spain). RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop ND-2000C, Thermo Fisher Scientific, Madrid, Spain). Two µg of total RNA from brain and eye were reverse transcribed into cDNA while using the M-MLV reverse transcriptase first strand cDNA synthesis kit (Promega Biotech Ibérica S.L.) following the manufacturer's instructions, and while using a mixture (3:1, *v/v*) of random primers and anchored oligo $(dT)_{15}$ primer (Promega Biotech Ibérica S.L.). Cloning of the *elovl4* full-length cDNA was carried out using PCR-based methodologies and brain-eye mix (1:1) cDNA as template. For *Sa elovl4* genes, degenerated primers UNIelovl4a-F/UNIelovl4a-R (*elovl4a*) and UNIelovl4b-F/UNIelovl4b-R (*elovl4b*), which were designed on conserved regions of teleost *elovl4a* and *elovl4b* orthologs available in the GenBank database, were used for amplification of the first fragment of the *Sa* putative *elovl4a* and *elovl4b* sequences with the PCR conditions that are shown in

the Table 3.1. The PCR fragments were then purified while using the Illustra GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare, Barcelona, Spain) and sequenced at least two times (DNA Sequencing Service, IBMCP-UPV, Valencia, Spain).

Two-round (nested) Rapid Amplification of cDNA ends (RACE) PCR were performed using the FirstChoice® RLM-RACE kit (Ambion, Life Technologies, Madrid, Spain) on each 3′ and 5′ RACE cDNA synthesized from *Sa* brain and eye RNA following the manufacturer's instructions in order to obtain the full-length ORF sequences. All of the primers used and PCR conditions are shown in Table 3.1. Potential positive fragments were cloned into pGEM-T Easy cloning vector (Promega Biotech Ibérica S.L.), while using GoTaq DNA polymerase (Promega Biotech Ibérica S.L.), and ligated with T4 DNA ligase (Promega Biotech Ibérica S.L.). The plasmid preparations were purified using the GenElute™ Plasmid Mini-prep Kit (Sigma-Aldrich, Madrid, Spain), and sequenced as described above. Two putative *elovl4* sequences were thus obtained and deposited in the GenBank database as gb|MK610320 (*elovl4a*) and gb|MK610321 (*elovl4b*). All primers used in this assay were designed using Primer3 software (http://primer3.sourceforge.net) (Rozen and Skalestky, 2000).

In the case of Senegalese sole, partial sequences were searched by gene annotation in the SoleaDB transcriptomic database (http://www.scbi.uma.es/soleadb)

version 4.1 for *Ss* global assembly and then grouped and assembled *in silico* while using the BioEdit Sequence Alignment Editor (BioEdit v7.0.9; Tom Hall, North Carolina State University, Raleigh, NC, USA). A BLAST search was performed in the National Center for Biotechnology Information (NCBI) online database (http://www.ncbi.nlm.nih.gov/) to compare with orthologs in other fish and lower vertebrate species and identify the ORF and untranslated regions (UTR) of the sequences. In order to obtain the full-length ORF sequences, given that 3' ends were missing but not 5' ends, two-round (nested) RACE-PCR were performed using the FirstChoice® RLM-RACE kit (Ambion, Life Technologies, Madrid, Spain) on 3′ RACE cDNA synthesized, as described above, from a 1:1 mix of Senegalese sole brain and eye RNA. The DNA fragments, which were amplified by PCR using GoTaq DNA polymerase (Promega Biotech Ibérica S.L.), were cloned into pGEM-T Easy (Promega Biotech Ibérica S.L.), purified using the GenElute™ Plasmid Miniprep Kit (Sigma–Aldrich), and then sequenced (DNA Sequencing Service, IBMCP-UPV), as above. Table 3.1 illustrates the primers used and PCR conditions. Two putative *elovl4* sequences were thus obtained and deposited in the GenBank database as gb|MN164537 (*elovl4a*) and gb|MN164625 (*elovl4b*).

Table 3.1. Nucleotide sequences of primers (Forward: F; Reverse: R) used for DNA open reading frame (ORF) cloning of *Sparus aurata* and *Solea senegalensis elovl4a* and *elovl4b*.

Sparus aurata					
Aim	**Primer**	**Sequence (5'-3')**	**Ta**	**PCR cycles**	**Extension time**
First fragment	UNIelovl4a-F	TGATGGACAACCCCCTGC	57 °C	35	1 min
	UNIelovl4a-R	GCAGATGAGGGAGTAGTGCAT	57 °C	35	1 min
	UNIelovl4b-F	ATGGAGCCTTACTATAGCAGAC	55 °C	35	1 min
	UNIelovl4b-R	GCGAAGAGGATGATGAAGGT	55 °C	35	1 min
5' RACE PCR	SaE4a-5R-R1	TTCTTCATGTACTTGGGCCC	60 °C	32	2 min 30 s
	SaE4a-5R-R2	AGAGGAACAGCAGGTAGGAGG	60 °C	32	2 min 30 s
	SaE4b-5R-R1	AGGTACAGGCAGCTGATGG	58 °C	32	2 min 30 s
	SaE4b-5R-R2	GAGATGACATCATGGGCCA	60 °C	32	2 min 30 s
3' RACE PCR	SaE4a-3R-F1	GTGGACCCAAGATCCAGAAG	60 °C	32	2 min 30 s
	SaE4a-3R-F2	TGTCCCTCTACGTCAACTGC	60 °C	32	2 min 30 s
	SaE4b-3R-F1	TACCTCACCATCATCCAGATG	58 °C	32	2 min 30 s
	SaE4b-3R-F2	CTCTACACAGGCTGCCCATT	60 °C	32	2 min 30 s
ORF Cloning	SaE4a-U-F1	GATCTTTAAAGCGCCGACAC	56 °C	32	2 min 40 s
	SaE4a-U-R1	TCCGGCTAAATCTTCCTCAA	56 °C	32	2 min 40 s
	SaE4a-V-F2	CCCGAATTCACCATGGAGATTGTCACACA	60 °C	32	2 min
	SaE4a-V-R2	CCGCTCGAGCTCTAATCTCTTTTAGCCCTT	60 °C	32	2 min
	SaE4b-U-F1	AATCGAGACCAAAGGCAGAG	56 °C	32	2 min 40 s
	SaE4b-U-R1	CTCTGTTAATCGCCGAGCAC	56 °C	32	2 min 40 s
	SaE4b-V-F2	CCCGAATTCACCATGGAGGTTGTAACACA	60 °C	32	2 min
	SaE4b-V-R2	CCGCTCGAGCCTCTTCCTTCTTTACTCCC	60 °C	32	2 min
Solea senegalensis					
Aim	**Primer**	**Sequence (5'-3')**	**Ta**	**PCR cycles**	**Extension time**
3' RACE PCR	SsE4a-3R-F1	GGAGGAGAAAGAGGAAAGG	60 °C	35	2 min 30 s
	SsE4a-3R-F2	GAAAGGAAGAGCTAAAAGAGA	60 °C	35	2 min 30 s
	SsE4b-3R-F1	CGGTCACCTTCATCATCCTC	60 °C	35	2 min 30 s
	SsE4b-3R-F2	ATGCCTTCCTACACCCAGAA	60 °C	35	2 min 30 s
ORF Cloning	SsE4a-U-F1	ACTGGATCACGACCACAACC	55 °C	32	2 min 15 s
	SsE4a-U-R1	TCCCAACACAGGCACATCTC	55 °C	32	2 min 15 s
	SsE4a-V-F2	CCCAAGCTTACCATGGAGATTGTCACACATTTA	55 °C	32	2 min
	SsE4a-V-R2	CCGCTCGAGTTAATCTCTTTTAGCTCTTCCTTTC	55 °C	32	2 min
	SsE4b-U-F1	CGGGGAGGAGGAGAAGAAGA	55 °C	32	2 min 15 s
	SsE4b-U-R1	AGCAATCCCCTTGACCGTTT	55 °C	32	2 min 15 s
	SsE4b-V-F2	CCCAAGCTTACCATGGAGGTTGTAACACATTTTG	55 °C	32	2 min
	SsE4b-V-R2	CCGCTCGAGTTACTCTCTTTTGGCTCTTCCTT	55 °C	32	2 min

PCR parameters, annealing temperatures (Ta), number of cycles (PCR cycles) and extension time, are shown. Restriction sites (*Eco*RI and *Xho*I for *S. aurata*; *Hin*dIII and *Xho*I for *S. senegalensis)* in primers used for cloning into yeast expressions vector pYES2 are underlined.

3.2.2. Sequence and phylogenetic analysis

The BLAST sequence analysis of NCBI was used for sequence alignment and phylogenetic analysis. The aa sequences that were deduced from the nucleotide sequences of the *Sa* Elovl4a (gb|QES86604.1), *Ss* Elovl4a (gb|QGA31141.1), *Sa* Elovl4b (gb|QES86605.1), and *Ss* Elovl4b (gb|QGA31140.1), were aligned using the ClustalW tool (BioEdit v7.0.9). The percentage of identity among aa sequences were obtained by comparison of Elovl4a and Elovl4b from both species by using Standard Protein BLAST (NCBI). The phylogenetic tree was constructed while using a total of 31 aa sequences, including the herein characterized *Sa* and *Ss* Elovl4 enzymes and other fish and vertebrate Elovl4, Elovl2, and Elovl5 sequences, while using the Maximum Likelihood method and the JTT matrix-based model (Jones *et al*., 1992). Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping through 1000 iterations. Phylogenetic analyses were conducted in MEGA X (Kumar *et al*., 2018).

3.2.3. Functional characterization of Sa and Ss Elovl4 isoforms

The *Sa* and *Ss* putative Elovl4 elongases were functionally characterized by determining the FA profiles of *Saccharomyces cerevisiae* (*S.c.* EasyComp™ Transformation Kit; Thermo Fisher Scientific) that were transformed with pYES2 yeast expression vector (Thermo Fisher Scientific) containing the putative *elovl4* as

inserts, and grown in the presence of potential FA substrates. Briefly, PCR fragments corresponding to the ORF of the *Sa* and *Ss elovl4a* and *elovl4b* were amplified by nested PCR from a mixture of cDNA (brain and eyes) while using the high fidelity *Pfu* DNA polymerase (Promega Biotech Ibérica S.L.) with primers containing restriction sites, corresponding to *Eco*RI (forward) and *Xho*I (reverse) for the *Sa elovl4* genes, and *Hin*dIII (forward) and *Xho*I (reverse) for the *Ss elovl4* genes (Table 3.1). The obtained DNA fragments were purified, as described above, digested with the appropriate restriction enzymes (New England Biolabs Ltd., Hitchin, United Kingdom), and then ligated into a similarly restricted pYES2 yeast expression vector. The purified plasmids (GenElute™ Plasmid Miniprep kit, Sigma-Aldrich) containing the putative *elovl4* ORF sequences were used to transform *S. cerevisiae* (strain InvSc1) competent cells. The transformation and selection of yeast with recombinant pYES2-*elovl4* or empty pYES2 (control) plasmids, and yeast culture were performed as described in detail previously (Jin *et al*., 2017).

Cultures of recombinant yeast were grown in *S. cerevisiae* minimal medium minus uracil (SCMM^{ura}, Sigma-Aldrich) broth supplemented with one of the following PUFA substrates: C_{18} (18:4n-3 and 18:3n-6), C_{20} (20:5n-3 and 20:4n-6), and C_{22} (22:5n-3, 22:6n-3 and 22:4n-6), in order to analyze the roles of *Sa* and *Ss* Elovl4 enzymes in VLC-PUFA biosynthesis. PUFA substrates were used at final concentrations 0.5 mM (C_{18}), 0.75 mM (C_{20}), and 1 mM (C_{22}) to compensate for

differential uptake related to FA acyl chain length (Monroig *et al*., 2010). Each PUFA substrate was tested once (n = 1). Recombinant yeast transformed with either pYES2 containing the corresponding *elovl4* ORF sequence or pYES2 with no insert (control) were grown in triplicate flasks (n = 3) in the absence of exogenously added FA to enable comparison of their long-chain (>C_{24}) saturated FA profiles to study the ability of *Sa* and *Ss* Elovl4 enzymes to biosynthesize VLC-SFA. After two days at 30 °C, the yeast cells were harvested by centrifugation, washed twice in double distilled H_2O, homogenized in 2:1 (*v/v*) chloroform:methanol containing 0.01 % (*w/v*) butylated hydroxytoluene (BHT, Sigma-Aldrich) as antioxidant, and then stored at -20 °C until further analysis. Yeasts that were transformed with pYES2 containing no insert were cultured under the same conditions as a control treatment. All FA substrates (>98-99 % pure) used for the functional characterization assays, except stearidonic acid (18:4n-3), were obtained from Nu-Chek Prep, Inc. (Elysian, MN, USA). Stearidonic acid (>99 % pure) and *S. cerevisiae* culture reagents, including galactose, nitrogen base, raffinose, tergitol NP-40, and uracil dropout medium, were obtained from Sigma-Aldrich.

3.2.4. Fatty acid analysis

The total lipids were extracted from yeast samples while using the method that was described by Folch *et al*. (1957). Fatty acid methyl esters (FAME) were

prepared, extracted, and purified, as described in detail in (Torres *et al*., 2020a). FAME were identified and quantified using a gas chromatograph (GC) coupled to a mass spectrometry (MS) detector, as described previously (Torres *et al*., 2020a). Briefly, the elongation of endogenous saturated FA with 24 carbons or longer was assessed by comparison of the areas of the fatty acids of control yeast with those of yeast that was transformed with each of the pYES2-*elovl4* plasmid constructs. The GC-MS was operated in the electron ionization (EI) single ion monitoring (SIM) mode. The 24:0, 26:0, 28:0, 30:0, 32:0, and 34:0 response values were obtained by using the *m/z* ratios 382.4, 410.4, 438.4, 466.5, 494.5, and 522.5, respectively. For VLC-PUFA analysis, the response values were obtained by using the *m/z* ratios 79.1, 108.1, and 150.1 in SIM mode (Agbaga *et al*., 2008; Garlito *et al*., 2019; Monroig *et al*., 2010). In this case, the elongation conversions of exogenously added PUFA were calculated as (area of first product and longer chain products/ (area of first product and longer chain products + substrate area)) × 100.

3.2.5. Tissue expression of elovl4 genes in gilthead seabream and Senegalese sole

3.2.5.1. Sample preparation

The samples for the tissue expression analysis of *elovl4* transcripts were obtained from three juveniles of gilthead seabream (14-17 cm; 40-60 g) and three Senegalese sole (16-19 cm; 60-80 g) that were maintained at the facilities of IATS-

CSIC, and fed on standard diets. While samples of selected tissues from the three fish of each species were used for qPCR, all of the tissues from a single fish were used for the RT-PCR study. All fish were anesthetized with 3-aminobenzoic acid ethyl ester (MS-222, 100 μg/mL) and then quickly sacrificed by cervical dislocation before sample collection of tissues, including brain mass, eye, gonad, liver, stomach, intestine, skin, white muscle, and red muscle. All of the tissue samples were immediately frozen and stored at -80 °C until required for RNA isolation. The total RNA was isolated from the tissues and cDNA samples were prepared from 2 μg of total RNA, as described above.

3.2.5.2. Gene expression analysis by reverse transcriptase PCR (RT-PCR)

The expression of *elovl4* isoforms in each tissue from one specimen of gilthead seabream and Senegalese sole was analyzed by reverse transcriptase PCR (RT-PCR) while using GoTag Polymerase (Promega Biotech Ibérica S.L.), using *18s ribosomal RNA* (*18s*) as a reference gene. PCR conditions consisted of an initial denaturing step at 95 °C for 2 min. followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 35 s, ending with a final extension at 72 °C for 5 min. Table 3.2 shows the primers used for RT-PCR on tissue cDNA samples. The RT-PCR products were assessed by gel electrophoresis and photodocumented while using UV light in a Gel Documentation System

Amersham Imager 600 (GE Healthcare UK Limited, Little Chalfont, UK). A random set of RT-PCR samples were purified and sequenced as above to confirm the identity of the amplicons.

3.2.5.3. Gene expression analysis by quantitative real-time PCR (qPCR)

The expression of *elovl4a* and *elovl4b* was analyzed by qPCR in tissues that showed a strong signal in RT-PCR analyses (brain, eye, and gonad), from three fish. Table 3.2 shows the primers used in qPCR analyses. The efficiency of the primer pairs was assessed through a standard curve that was obtained by serial dilutions of standard solutions of the studied genes with known copy numbers, which also allowed for the conversion of threshold cycle (Ct) values to copy numbers. The amplification was carried out, as previously described in (Torres *et al*., 2020a). Three potential reference genes (*β-actin*, *elongation factor 1α* and *18s rRNA*) were tested. After checking gene stability using the Genorm software (Vandesompele *et al*., 2002), *β-actin* was chosen for gene expression normalization. The gene expression results are given as mean normalized values ± standard deviation (SD) corresponding to the ratio between copy numbers of fatty acyl elongase (*elovl4a* and *elovl4b*) transcripts and copy numbers of the reference gene *β-actin* (*actb*).

Table 3.2 Primers used for reverse transcriptase PCR (RT-PCR) and real-time quantitative PCR (qPCR) of *Sparus aurata* and *Solea senegalensis* genes.

Sparus aurata						
Aim	**Transcript**	**Primer**	**Primer Sequence (5'–3')**	**Ta**	**Fragment**	**Accession No**
RT-PCR	*elovl4a*	F	GCCCAAGTACATGAAGAACAGAG	60 °C	563 bp	MK610320
		R	GGGAGTAGTGCATCCAGTGG			
	elovl4b	F	GTCAAGTACTCCAACGATGTCAA	60 °C	394 bp	MK610321
		R	GGAATGGGCAGCCTGTGT			
	18s	F	TCCTTTGATCGCTCTACCGT	60 °C	460 bp	AY993930.1
		R	TGCCCTCCAATTGATCCTCG			
qPCR	*elovl4a*	F	GCCCAAGTACATGAAGAACAGAG	60 °C	169 bp	MK610320
		R	ACCTGATGAGTCTGCTGGGG			
	elovl4b	F	GTCAAGTACTCCAACGATGTCAA	60 °C	247 bp	MK610321
		R	GAGAAGGTAGGTACACGAGT			
	Actb	F	TGCGTGACATCAAGGAGAAG	60 °C	190 bp	X89920
		R	AAGGAGCCATACCTCAGGAC			
Solea senegalensis						
Aim	**Transcript**	**Primer**	**Primer Sequence (5'–3')**	**Ta**	**Fragment**	**Accession No**
RT-PCR	*elovl4a*	F	TGCACTACTCCCTCATCTGC	60 °C	497 bp	MN164537
		R	TGAAAACAGCCACCTTAGGC			
	elovl4b	F	CCTCTGCCTTGTCCAGTTTC	60 °C	175 bp	MN164625
		R	TCCTTGACCCGTAGTTTAAC			
	18s	F	TCAGACCCAAAACCCATGCG	60 °C	464 bp	EF126042.1
		R	CCCGAGATCCAACTACGAGC			
qPCR	*elovl4a*	F	AGGTGAGGTAGGGCCTTGTT	60 °C	220 bp	MN164537
		R	CGGATTCCACCGACAAAAGT			
	elovl4b	F	CCTCTGCCTTGTCCAGTTTC	60 °C	175 bp	MN164625
		R	TCCTTGACCCGTAGTTTAAC			
	Actb	F	ACAATGAGCTGAGAGTCGCC	60 °C	132 bp	DQ485686
		R	ATGGGGGCGGTACATACAAC			

Sequences of primer pairs used (Forward: F; Reverse: R), annealing temperatures (Ta) of primer pairs, size of fragments produced, and accession number of the sequences used for primer design are shown.

3.2.6. Statistical analysis

The homogeneity of variances of the data that were associated to VLC-SFA (%) and tissue gene expression values, as determined by qPCR, were checked using Levene's test. Statistical differences were analyzed by one-way analysis of variance (ANOVA) ($p \leq 0.05$), followed by Tukey HSD *post-hoc* tests. The statistical software SPSS 26.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data.

3.3. Results

3.3.1. Elovl4 sequence and phylogenetic analysis

The *Sa* and *Ss elovl4a* ORF sequences have 969 base pairs (bp) and 960 bp, encoding putative proteins of 322 amino acids (aa) and 319 aa, respectively (**Figure 3.2**). On the other hand, *Sa* and *Ss elovl4b* ORF sequences contain 918 bp, encoding proteins of 305 aa (**Figure 3.2**). Elovl4 from both *Sa* and *Ss* contain the conserved histidine binding box motif (HXXHH), the predicted endoplasmic reticulum (ER) retention signal with arginine (R) and lysine (K) at the carboxyl end (RXKXX), as well as several regions with conserved motifs (**Figure 3.2**). This suggests that *Sa* and *Ss elovl4* cDNA both encode Elovl4a and Elovl4b enzymes, which have been deposited in Genbank under the following accession numbers: *Sa* Elovl4a (MK610320), *Sa* Elovl4b (MK610321), *Ss* Elovl4a (MN164537), and *Ss* Elovl4b (MN164625). We compared the deduced aa sequence of Elovl4a and Elovl4b from both species via BLASTp searches. The results revealed that *Sa* and *Ss* Elovl4a aa sequences are both 95 % identical. Likewise, both *Sa* and *Ss* Elovl4b aa sequences show 90 % identity with each other. The phylogenetic analysis showed that both Elovl4 sequences from each species form two separate clusters that include either Elovl4a or Elovl4b sequences from a range of teleosts (**Figure 3.3**). *Sa* and *Ss* Elovl4a both clustered together, while *Sa* and *Ss* Elovl4b clustered more closely with

A. schlegelii Elovl4b and Atlantic bluefin tuna (*Thunnus thynnus*) Elovl4b, respectively (**Figure 3.3**). All fish Elovl4 elongases grouped with mammalian and birds orthologs, and separately from other vertebrate Elovl proteins, such as Elovl2 and Elovl5.

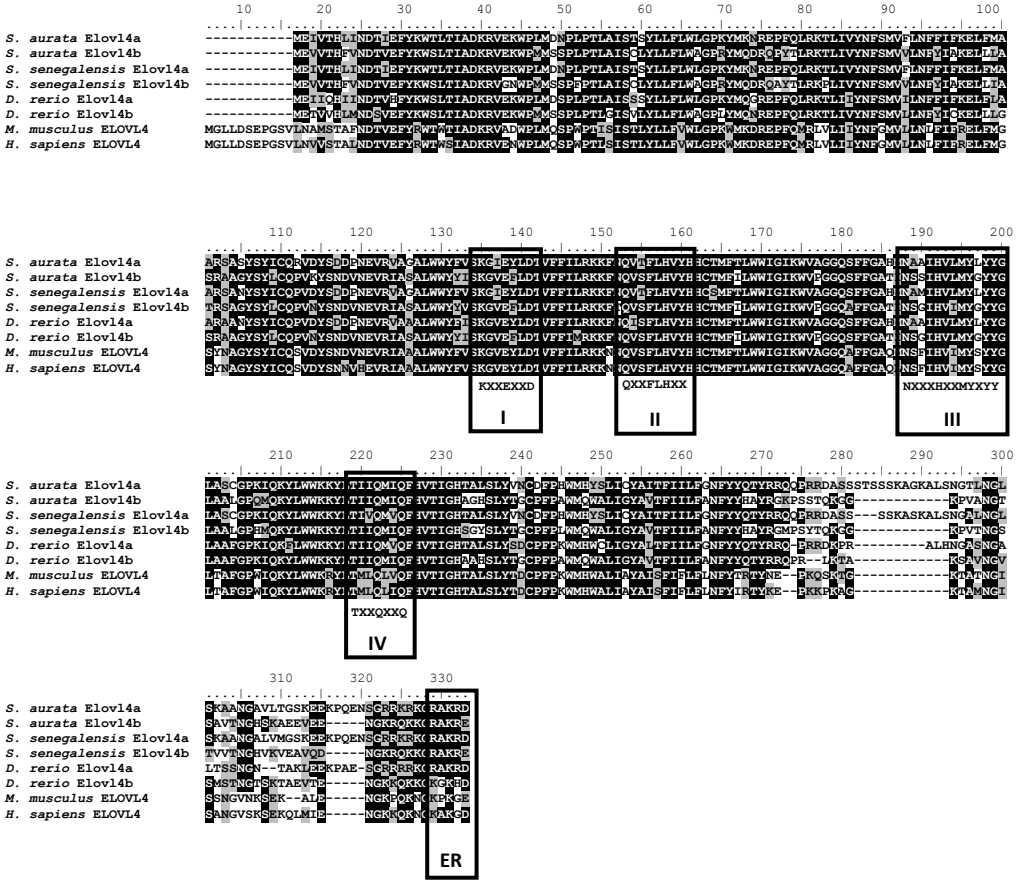

Figure 3.2. ClustalW amino acid alignment of the deduced *Sparus aurata* Elovl4a (gb|QES86604.1), *Solea senegalensis* Elovl4a (gb|QGA31141.1), *Sparus aurata* Elovl4b (gb| QES86605.1), and *Solea senegalensis* Elovl4b (gb| QGA31140.1). Identical residues are shaded black and similar residues are shaded grey. The four (I-IV) conserved motifs of Elovl enzymes: I (KXXEXXDT), II (QXXFLHXXHH), III (NXXXHXXMYXYY) and IV (TXXQXXQ), as well as the endoplasmic reticulum (ER) retrieval signal (RXKXX) at the C-terminus, are indicated by squares.

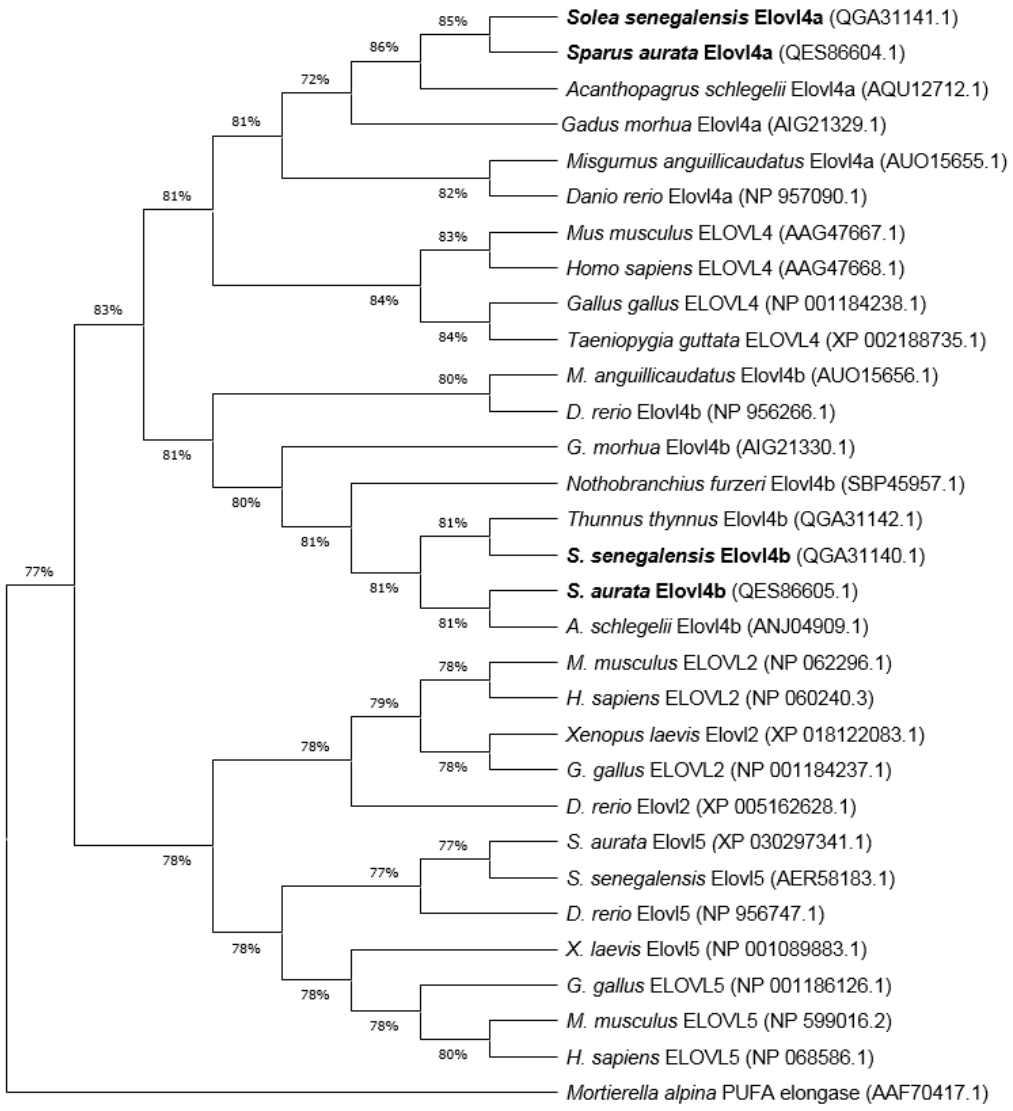

Figure 3.3. Phylogenetic tree comparing *Sparus aurata* and *Solea senegalensis* Elovl4a and Elovl4b proteins (highlighted in bold) with Elovl2, Elovl4 and Elovl5 proteins from other vertebrates. The tree was constructed while using the Maximum Likelihood method and Jones-Taylor-Thornton (JTT) matrix-based model. The numbers in branches represent the frequencies (%) of each node after 1000 iterations by bootstrapping. The *Mortierella alpina* PUFA elongase was included in the analysis as an outgroup, to construct the rooted tree.

3.3.2. Functional characterization of Elovl4a and Elovl4b

The *Sa* and *Ss* putative Elovl4 elongases were functionally characterized in yeast *S. cerevisiae* (strain InvSc1). Firstly, we investigated the ability of *Sa* and *Ss* Elovl4 to elongate VLC-SFA by comparing the SFA profiles of yeast that was transformed with the yeast expression vector pYES2 containing the *elovl4* coding regions, with those of control yeast transformed with empty pYES2, after incubation in the absence of exogenously added FA. The results confirmed that *Sa* and *Ss* Elovl4 isoforms are both involved in the biosynthesis of VLC-SFA (Tables 3.3 and 3.4). More specifically, *Sa elovl4* transformed yeast showed a different ($p \leq 0.05$) profile of SFA $\geq C_{24}$ when compared to control yeast, with decreased contents of 26:0 and increased levels of 28:0, 30:0, and 32:0 (Table 3.3). These results suggest that 26:0 is an important substrate for both *Sa* Elovl4 proteins, with Elovl4a appearing as the most active isoform, since the contents of all detected VLC-SFA longer than 26:0 were significantly higher as compared to the control (Table 3.3). On the other hand, *Ss elovl4* transformed yeast showed increased levels of 24:0 and 26:0, along with decreased levels of 28:0, 30:0, and 32:0 (Table 3.4). Therefore, the results suggest that the *Ss* Elovl4 proteins are involved in the biosynthesis of VLC-SFA up to 26:0, for which they use $<C_{24}$ fatty acids as elongation substrates.

Table 3.3. Functional characterization of *Sparus aurata* Elovl4 elongases: role in the biosynthesis of very long-chain saturated fatty acids (FA).

FA	Elovl4a	Elovl4b	Control
24:0	14.6[a]	11.3[a]	12.1[a]
26:0	49.5[b]	68.2[a]	75.0[a]
28:0	20.8[c]	14.1[b]	8.6[a]
30:0	11.0[b]	4.5[a]	2.7[a]
32:0	3.3[b]	1.5[a]	1.0[a]
34:0	0.7[a]	0.3[a]	0.3[a]

Results are expressed as area percentage (%) of total saturated FA $\geq C_{24}$ found in yeast transformed with either pYES2 containing the *elovl4* coding regions or empty pYES2 vector (control) (n = 3). Different superscripts (a, b and c) denote significant differences in each row, among area percentages of each saturated FA (one way-ANOVA and Tukey test, $p \leq 0.05$).

Table 3.4. Functional characterization of *Solea senegalensis* Elovl4 elongases: role in the biosynthesis of very long-chain saturated fatty acids (FA).

FA	Elovl4a	Elovl4b	Control
24:0	9.2[b]	9.5[b]	6.1[a]
26:0	72.1[b]	81.2[b]	58.3[a]
28:0	11.9[b]	5.7[c]	21.7[a]
30:0	5.5[b]	2.9[b]	11.5[a]
32:0	1.4[ab]	0.7[b]	2.4[a]
34:0	0.0[a]	0.0[a]	0.0[a]

Results are expressed as area percentage (%) of total saturated FA $\geq C_{24}$ found in yeast transformed with either pYES2 containing the *elovl4* coding regions or empty pYES2 vector (control) (n = 3). Different superscripts (a, b and c) denote significant differences in each row, among area percentages of each saturated FA (one way-ANOVA and Tukey test, $p \leq 0.05$).

Yeast transformed with both Elovl4 were incubated with C_{18} (18:4n-3 and 18:3n-6), C_{20} (20:5n-3 and 20:4n-6), and C_{22} (22:5n-3, 22:6n-3 and 22:4n-6) PUFA substrates in order to test the role that *Sa* and *Ss* Elovl4 elongases play in VLC-PUFA biosynthesis. It is commonly assumed that the fatty acid composition of yeast

transformed with empty pYES2 (control) only consists of saturated and monounsaturated fatty acids, together with the corresponding exogenously added PUFA, since it is well established that *S. cerevisiae* possesses no PUFA elongation activity (Agaba *et al*., 2004). The chromatographic analyses of Elovl4-transformed yeast revealed that *Sa* Elovl4a elongated all n-6 PUFA substrates, i.e., 18:3n-6, 20:4n-6, and 22:4n-6 (Table 3.5), as well as n-3 PUFA substrates (18:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3) (Table 3.5). *Sa* Elovl4b also elongated n-6 PUFA substrates, but showed particularly high affinity towards n-3 PUFA substrates, especially to elongate 22:6n-3 (DHA) substrates to 32:6n-3 (Table 3.5). Moreover, *Sa* Elovl4 elongases were both able to convert 20:5n-3 or 22:5n-3 to 24:5n-3, an intermediate substrate for DHA synthesis via the Sprecher pathway (Sprecher, 2000).

Table 3.5. Functional characterization of the *Sparus aurata* Elovl4a and Elovl4b elongases by heterologous expression in the yeast *Saccharomyces cerevisiae*.

FA Substrate	Product	Elovl4a	Elovl4b
		% Conversion	% Conversion
18:4n-3	20:4n-3	2.5	2.7
	22:4n-3	9.7	12.5
	24:4n-3	5.6	49.9
	26:4n-3	n.d.	65.6
	28:4n-3	n.d.	n.d.
	30:4n-3	n.d.	n.d.
	32:4n-3	n.d.	n.d.
	34:4n-3	n.d.	n.d.
	36:4n-3	n.d.	n.d.
18:3n-6	20:3n-6	2.6	2.1
	22:3n-6	21.6	9.6
	24:3n-6	52.5	n.d.
	26:3n-6	57.1	n.d.

	28:3n-6	64.8	n.d.
	30:3n-6	90.0	n.d.
	32:3n-6	84.1	n.d.
	34:3n-6	41.3	n.d.
	36:3n-6	n.d.	n.d.
20:5n-3	22:5n-3	5.8	9.1
	24:5n-3	17.2	33.3
	26:5n-3	20.0	57.8
	28:5n-3	n.d.	86.8
	30:5n-3	n.d.	97.7
	32:5n-3	n.d.	72.7
	34:5n-3	n.d.	8.1
	36:5n-3	n.d.	n.d.
20:4n-6	22:4n-6	10.9	8.9
	24:4n-6	31.0	30.2
	26:4n-6	37.1	55.9
	28:4n-6	39.0	81.0
	30:4n-6	88.6	37.8
	32:4n-6	83.6	n.d.
	34:4n-6	73.7	n.d.
	36:4n-6	11.4	n.d.
22:5n-3	24:5n-3	3.4	12.6
	26:5n-3	19.8	52.2
	28:5n-3	26.0	86.3
	30:5n-3	85.6	96.5
	32:5n-3	74.2	64.4
	34:5n-3	63.0	5.3
	36:5n-3	n.d.	n.d.
22:4n-6	24:4n-6	8.2	10.4
	26:4n-6	35.1	43.1
	28:4n-6	45.5	71.8
	30:4n-6	90.8	83.0
	32:4n-6	78.7	19.5
	34:4n-6	54.6	n.d.
	36:4n-6	7.2	n.d.
22:6n-3	24:6n-3	0.4	1.8
	26:6n-3	n.d.	100
	28:6n-3	n.d.	100
	30:6n-3	n.d.	40.2
	32:6n-3	n.d.	61.3
	34:6n-3	n.d.	n.d.
	36:6n-3	n.d.	n.d.

The data are presented as the percentage conversions of polyunsaturated fatty acid (FA) substrates (*n* = 1). Individual conversions were calculated according to the formula (area of first product and longer chain products/ (area of first product and longer chain products + substrate area)) × 100. n.d.: not detected.

For Senegalese sole, both Elovl4 elongases presented the capability to elongate PUFA substrates from the n-3 (18:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3) and n-6 (18:3n-6, 20:4n-6, and 22:4n-6) series, to longer chain FA of up to C_{34} (Table 3.6). As described above for *Sa*, *Ss* Elovl4b was able to convert 22:6n-3 (DHA) to VLC-PUFA of up to C_{32}, an elongation capacity not exhibited by the *Ss* Elovl4a. It is noteworthy that *Ss* Elovl4b, and to a lesser extent Elovl4a, showed high capacity to elongate 20:5n-3 (EPA) to 22:5n-3 (Table 3.6), a key step that is required for DHA synthesis via the Δ4 pathway.

Table 3.6. Functional characterization of the *Solea senegalensis* Elovl4a and Elovl4b elongases by heterologous expression in the yeast *Saccharomyces cerevisiae*.

FA substrate	Product	Elovl4a	Elovl4b
		% Conversion	% Conversion
18:4n-3	20:4n-3	4.5	8.1
	22:4n-3	19.6	41.2
	24:4n-3	39.5	79.0
	26:4n-3	39.6	95.3
	28:4n-3	100	96.8
	30:4n-3	100	98.7
	32:4n-3	65.4	65.7
	34:4n-3	n.d.	1.7
	36:4n-3	n.d.	n.d.
18:3n-6	20:3n-6	4.6	6.2
	22:3n-6	38.6	40.8
	24:3n-6	66.2	66.0
	26:3n-6	65.3	89.1
	28:3n-6	100	91.9
	30:3n-6	55.0	90.4
	32:3n-6	62.7	17.8
	34:3n-6	n.d.	n.d.
	36:3n-6	n.d.	n.d.
20:5n-3	22:5n-3	12.1	30.9
	24:5n-3	31.8	75.1
	26:5n-3	35.7	87.4

	28:5n-3	100	96.9
	30:5n-3	50.0	98.9
	32:5n-3	33.7	82.9
	34:5n-3	38.2	14.5
	36:5n-3	n.d.	n.d.
20:4n-6	22:4n-6	18.1	33.1
	24:4n-6	49.9	73.4
	26:4n-6	56.7	85.1
	28:4n-6	65.2	94.3
	30:4n-6	95.2	95.9
	32:4n-6	84.9	51.8
	34:4n-6	25.3	2.7
	36:4n-6	n.d.	n.d.
22:5n-3	24:5n-3	7.8	44.3
	26:5n-3	33.9	87.9
	28:5n-3	51.2	97.0
	30:5n-3	92.3	99.0
	32:5n-3	27.4	82.5
	34:5n-3	32.4	16.2
	36:5n-3	n.d.	n.d.
22:4n-6	24:4n-6	13.5	37.2
	26:4n-6	58.3	85.5
	28:4n-6	71.8	94.5
	30:4n-6	94.5	96.3
	32:4n-6	21.6	53.9
	34:4n-6	25.9	5.0
	36:4n-6	n.d.	n.d.
22:6n-3	24:6n-3	0.6	5.1
	26:6n-3	n.d.	100
	28:6n-3	n.d.	100
	30:6n-3	n.d.	100
	32:6n-3	n.d.	22.3
	34:6n-3	n.d.	n.d.
	36:6n-3	n.d.	n.d.

Data are presented as the percentage conversions of polyunsaturated fatty acid (FA) substrates (n = 1). Individual conversions were calculated according to the formula (area of first product and longer chain products/ (area of first product and longer chain products + substrate area)) × 100**.** n.d.: not detected.

3.3.3. Tissue expression of elovl4 genes

The tissue distribution of the two *elovl4* mRNAs in gilthead seabream and Senegalese sole was analyzed by reverse transcription polymerase chain reaction (RT-PCR). In both species, *elovl4a* and *elovl4b* transcripts appear to be present in most of the analyzed tissues. In tissues of the gastrointestinal tract, liver, skin, or muscle, expression was only found with low signal intensity (**Figures 3.4 A-B**). Although comparisons of transcript levels from RT-PCR analyses have to be made cautiously, for gilthead seabream, strong expression signals were found in the brain and gonad for *elovl4a,* and brain, eye, and gonad for *elovl4b* (**Figure 3.4 A**). In the case of Senegalese sole, strong *elovl4a* expression was detected in the brain, with eye and brain having a high signal of *elovl4b* expression (**Figure 3.4 B**).

Selected tissues of both fish species were then analyzed by real-time quantitative reverse transcriptase PCR (qPCR) in order to obtain quantitative values of *elovl4a* and *elovl4b* expression (**Figures 3.4 C-F**). For gilthead seabream, significant differences ($p \leq 0.05$) were found in *elovl4a* expression between all tissues, with the highest levels of mRNA in brain, followed by eye, and finally gonad (**Figure 3.4 C**). For *elovl4b*, the highest expression was found in eye, while the brain and gonad showed significantly lower expression values ($p \leq 0.05$) (**Figure 3.4 E**). Similarly, a differential expression of *elovl4* genes was detected in the tissues of Senegalese sole ($p \leq 0.05$). For *elovl4a*, significantly higher expression was found in brain, with a

lower expression being measured in the eye and gonad (**Figure 3.4 D**). In the case of *elovl4b*, the highest expression levels were detected in eye, with brain and gonad having significantly lower expression levels (**Figure 3.4 F**).

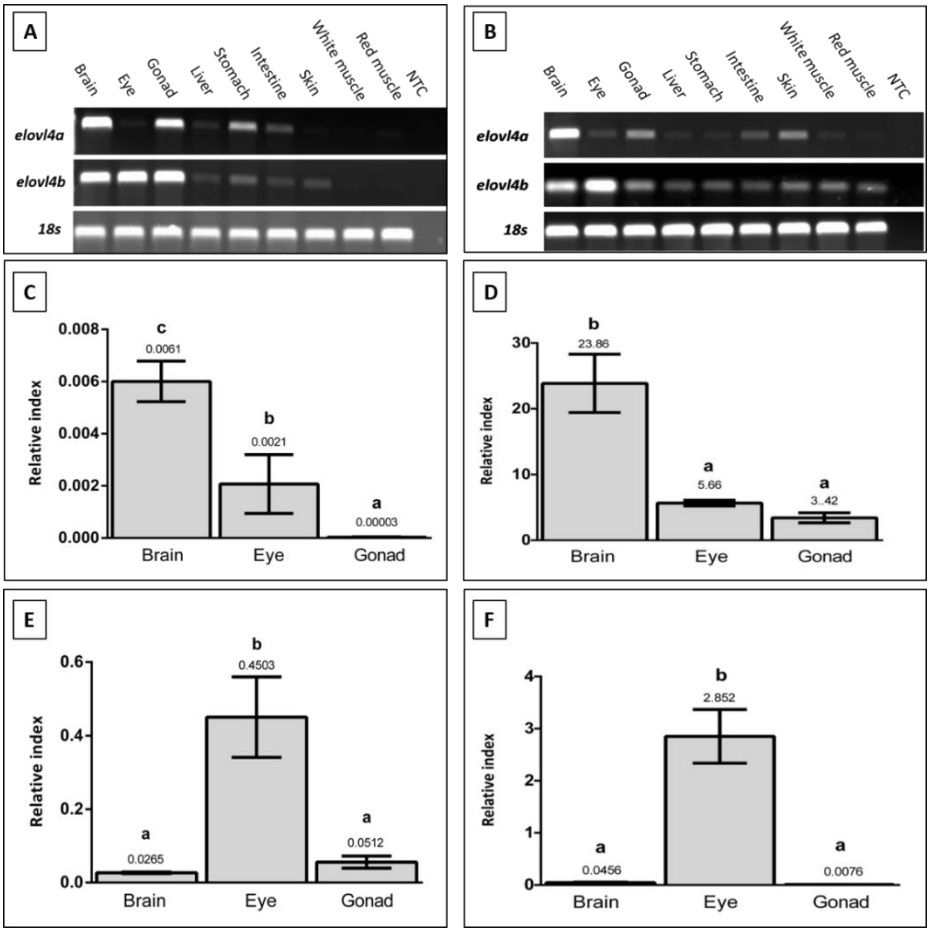

Figure 3.4. Tissue distribution of *elovl4a* and *elovl4b* transcripts in *Sparus aurata* (**A**) and *Solea senegalensis* (**B**) determined by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) (n = 1 fish). Expression of housekeeping gene *18s* is also shown. Expression in selected tissues of *Sparus aurata elovl4a* (**C**) *Solea senegalensis elovl4a* (**D**), *Sparus aurata elovl4b* (**E**) and *Solea senegalensis elovl4b* (**F**) transcripts were also determined by qPCR. The results, shown as relative index, are β-actin normalized values (gene copy number/*β-actin* copy number). Bars represent means and standard deviations (n = 3 fish). Different letters denote significant differences (ANOVA and Tukey HSD test, $p \leq 0.05$) among tissues.

3.4. Discussion

Sequence analyses revealed that the investigated predicted Elovl4 proteins contain all the characteristic domains of vertebrate Elovl4 family members (Cook and Mcmaster, 2002; Marchler-Bauer *et al*., 2017), including the ER retention signal (RXKXX), the histidine box (HXXHH), which is involved in the electron transfer process during fatty acid elongation (Jakobsson *et al*., 2006), and other transmembrane domains, similarly to what has been described in other fish species (Betancor *et al*., 2020; Carmona-Antoñanzas *et al*., 2011; Jin *et al*., 2017; Kabeya *et al*., 2015; Li *et al*., 2017a, b; Monroig *et al*., 2010, 2011; Oboh *et al*., 2017; Zhao *et al*., 2019). Furthermore, phylogenetic analysis confirmed the existence of two isoforms of *Sa* and *Ss* Elovl4, which clustered, together with corresponding Elovl4 orthologs from other teleosts, into different branches, thus confirming that the described Elovl4 isoforms are true orthologs of the Elovl4a and Elovl4b proteins that are present in teleosts (Castro *et al*., 2016). The conservation of both Elovl4 isoforms in fish genomes (Castro *et al*., 2016; Jin *et al*., 2017; Kabeya *et al*., 2015; Monroig *et al*., 2010; Oboh *et al*., 2017; Yan *et al*., 2018) and their clear segregation into separate clusters points towards a likely functional specialization of these proteins in teleosts (Monroig *et al*., 2011; Zhao *et al*., 2019), which we aimed to further elucidate in this study by functionally characterizing the two isoforms in two new fish species with diverse life histories, dietary habits, and notably different LC-

PUFA biosynthesis mechanisms (Agaba *et al*., 2005; Morais *et al*., 2012; Seiliez *et al*., 2003; Zheng *et al*., 2004).

Our results support the notion that both isoforms can participate in VLC-SFA elongation, as suggested in previous studies with other fish species including *D. rerio* (Monroig *et al*., 2010), *C. gariepinus* (Oboh *et al*., 2017) and Atlantic salmon (*Salmo salar*) (Carmona-Antoñanzas *et al*., 2011). Nevertheless, Elovl4a seems to be more efficient than Elovl4b at elongating VLC-SFA, similarly to what was reported in *D. rerio* (Monroig *et al*., 2010). This was particularly evident in *Sa*, where the comparison of the SFA profile of yeast transformed with *elovl4a* or *elovl4b* with that of control yeast revealed that Elovl4b was only active in the elongation step from 26:0 to 28:0, whereas Elovl4a was clearly able to significantly elongate SFA substrates from 26:0 up to 32:0. The functional characterization of *Ss* Elovl4 enzymes showed some differences with respect to *Sa*, particularly concerning the preferred fatty acid substrates. Whereas the *Sa* Elovl4 isoforms have 26:0 as the most preferred precursor for VLC-SFA biosynthesis, $<C_{24}$ saturated fatty acids appear to be more adequate for the *Ss* Elovl4 isoforms. This could be related to differences in VLC-SFA requirements between the two fish species (Torres *et al*., 2020a), but further studies are necessary in order to clearly establish this.

Functional analyses of Elovl4a and Elovl4b confirmed that both proteins actively participate in the biosynthesis of either n-6 or n-3 VLC-PUFA, from n-6 and

n-3 PUFA substrates, in the two studied fish species. These results are in agreement with Elovl4 functional characterization studies in other fish species, which showed similar elongation capabilities (Betancor *et al*., 2020; Carmona-Antoñanzas *et al*., 2011; Jin *et al*., 2017; Li *et al*., 2017a, b; Monroig *et al*., 2010; Oboh *et al*., 2017), consistent with the functionality that is described in mammals (Agbaga *et al*., 2008) and other aquatic organisms, such as common octopus (*Octopus vulgaris*) (Monroig *et al*., 2017). However, intra- and inter-specific differences were found in the efficiency of the different Elovl4 isoforms to biosynthesize VLC-PUFA. In this respect, *Sa* Elovl4a showed a clearly higher affinity towards the elongation of n-6 PUFA substrates, while *Sa* Elovl4b was particularly active towards n-3 PUFA substrates. On the other hand, only Elovl4b was able to elongate DHA, up to 34:6n-3. Similar results were obtained in Senegalese sole, where both of the isoforms were active towards n-6 or n-3 PUFA substrates, in this case with less clear differences in terms of substrate preference, but only Elovl4b showed activity towards DHA.

Interestingly, although fish Elov4b seem to have a predominant role in the DHA biosynthesis pathway (Betancor *et al*., 2020; Jin *et al*., 2017; Oboh *et al*., 2017), both *Sa* Elovl4 isoforms had the ability to elongate 20:5n-3 and 22:5n-3 to 24:5n-3, which is a key intermediate FA in the biosynthesis of DHA via the Δ6 "Sprecher pathway" (Sprecher, 2000) occurring in this species (Seiliez *et al*., 2003; Zheng *et al*., 2004). Similarly, both *Ss* Elovl4 isoforms, although Elovl4b more

prominently, had the capacity to produce 22:5n-3 from 20:5n-3. This is a key substrate for DHA biosynthesis via the Δ4-desaturation pathway that was carried out by *Ss* Fads2 (Morais *et al*., 2012). This redundancy in an activity that is central for DHA biosynthesis (Jin *et al*., 2017; Kabeya *et al*., 2015; Li *et al*., 2017b; Monroig *et al*., 2010, 2011; Yan *et al*., 2018) highlights the essentiality of this compound for the correct development and survival of marine fish (Monroig *et al*., 2018). It is well known that the correct biosynthesis of DHA is crucial for the normal development of the fish cognitive system, especially during early stages, when its deficiency can cause visual and/or neural damage (Benitez-Santana *et al*., 2007; Mourente, 2003). Moreover, it is highly conceivable that the conservation of two Elovl4 enzymes with the ability to elongate 22:5n-3 and 24:5n-3 can confer a substantial adaptive advantage in marine fish species that have lost the *elovl2* gene during evolution (Castro *et al*., 2016; Jin *et al*., 2017; Monroig *et al*., 2018).

It is also noteworthy that, similar to what has been described in zebrafish (Monroig *et al*., 2010), *Sa* and *Ss* Elovl4a elongases both showed low elongation activity from DHA to 24:6n-3. This could suggest that, as described in rat retinas (Suh and Clandinin, 2005), EPA and not DHA might be the preferred substrate for VLC-PUFA biosynthesis in fish. This assumes the formation of VLC-PUFA hexaenes from LC-PUFA pentaenes via Δ6 desaturation of 24:5n-3, which should only take place in the case of gilthead seabream, since Senegalese sole is believed to

lack this desaturation capacity in favour of a Δ4 Sprecher-independent pathway for DHA biosynthesis (Morais *et al*., 2012). Paradoxically, our functional results revealed a higher capacity for 24:5n-3 production in this latter species. On the other hand, similarly to what has been found in other teleosts (Betancor *et al*., 2020; Oboh *et al*., 2017; Jin *et al*., 2017), Elovl4b proteins in both species were able to elongate 24:6n-3 up to 32:6n-3, a VLC-PUFA found in retinal phosphatidylcholine in fish (Monroig *et al*., 2016; Garlito *et al*., 2019). Thus, this specific activity of Elovl4b proteins, along with the above-mentioned presence of 32:6n-3 in fish retina, is coherent with the tissue expression results obtained for both species, in which Elovl4b transcripts were mostly found in the eye suggesting that, similarly to what has been described in other teleosts, like *T. thynnus* (Betancor *et al*., 2020), *D. rerio* (Monroig *et al*., 2010), *A. schlegelli* (Jin *et al*., 2017), rainbow trout (*Oncorhynchus mykiss*) (Zhao *et al*., 2019), *S. salar* (Carmona-Antoñanzas *et al*., 2011), or orange-spotted grouper (*Epinephelus coioides*) (Li *et al*., 2017b); this is a major tissue for VLC-PUFA biosynthesis.

The quantitative expression results confirmed previous evidences of a differential *elovl4a* and *elovl4b* tissue-specific expression pattern (Torres *et al*., 2020a, b), with *elovl4a* being mostly expressed in fish brain (Monroig *et al*., 2010; Oboh *et al*., 2017; Jin *et al*., 2017; Zhao *et al*., 2019), and *elovl4b* in eye (Betancor *et al*., 2020; Carmona-Antoñanzas *et al*., 2011; Jin *et al*., 2017; Li *et al*., 2017b;

Monroig *et al*., 2010; Zhao *et al*., 2019). Therefore, in spite of the specific functions of VLC-PUFA still not being fully understood in vertebrates (Agbaga *et al*., 2010), and their identification being very scarce in fish (Garlito *et al*., 2019; Monroig *et al*., 2016), the results reported here in terms of *elovl4* tissue expression in these two fish species suggests a role of these enzymes in the local biosynthesis and the incorporation of VLC-FA in fish neural tissues. This is in agreement with what is known in mammals (Deák *et al*., 2019), in which VLC-PUFA are key functional components, essential for the development and cell protection, of neural tissues such as those found in retina or brain (Agbaga *et al*., 2008, 2010; Bazan, 2018). More specifically, certain VLC-PUFA are synthesized and esterified at the *sn-1* position of the glycerol backbone of phosphatidylcholine, which is then deposited in retinal photoreceptors, where it plays an important neuroprotective role (Agbaga *et al*., 2010; Deák *et al*., 2019; Poulos *et al*., 1988). Other VLC-SFA are mainly incorporated into sphingolipids in the central nervous system (Deák *et al*., 2019), playing a key role in the membrane fusion of synaptic vesicles occurring during neurotransmission processes (Hopiavuori *et al*., 2018, 2019).

The application of this knowledge is of special relevance during early larval development, particularly in species with high commercial interest for aquaculture production, as is the case of gilthead seabream and Senegalese sole, and it should be kept in mind in feeding protocols during hatchery rearing. Mammalian *elovl4*

expression is developmentally regulated in the brain, with expression peaking around the time of birth and falling as the brain matures (Mandal *et al*., 2004), thus pointing to a prominent role of this protein in neurogenesis (Deák *et al*., 2019). In fish species, it is equally likely that the optimal functioning of Elovl4 enzymes is particularly critical during early developmental stages, at a time when neural tissues are rapidly forming (Tocher, 1988, 2003), in order to ensure correct biosynthesis and tissue accumulation of VLC-PUFA products (Monroig *et al*., 2010, 2011; Torres *et al*., 2020a, b). Hence, not surprisingly, *elovl4* genes were found to be widely expressed in neural tissues (brain mass and eyes) during the embryonic phase of *D. rerio* (Monroig *et al*., 2010) and cobia (*Rachycentron canadum*) (Monroig *et al*., 2011). Moreover, as previously described (Torres *et al*., 2020a), retinogenesis in gilthead seabream and Senegalese sole larvae is clearly synchronized with an increase in expression of both *elovl4* genes. Consequently, as described in mammals (Bennett *et al*., 2014), alterations in VLC-PUFA biosynthesis could negatively impact visual acuity and disrupt brain functionality, jeopardizing the normal development of fish. Although neural and visual structures of newly hatched fish larvae are undeveloped, cones and rod cells differentiate quite early (Hu *et al*., 2018, Lim *et al*., 2014), and their correct development and functionality is determining for fish larvae to begin feeding exogenously (Turkmen *et al*., 2017) and, hence, for their survivability. This is particularly relevant in visual predators, such as gilthead

seabream and Senegalese sole, which previously showed a differential *elovl4* expression in larvae and post-larvae according to the VLC-PUFA putative needs associated with each life-stage and LC-PUFA dietary availability (Torres *et al*., 2020a, b). Finally, the results from the present study evidencing a low activity of Elovl4 on DHA and a higher activity on longer (26 and 28 C) substrates, which are, in turn, dependent on DHA, reinforce the idea that an appropriately high dietary supply of DHA is critical in early stages of fish larval life, not only "per se", i.e., related to the essential nature of this fatty acid on its own, but also as a bottleneck substrate for subsequent VLC-PUFA synthesis.

In view of the results that are presented here, we conclude that both gilthead seabream and Senegalese sole possess two distinct Elovl4-like elongases named Elovl4a and Elovl4b based on their homology to the zebrafish orthologs (Monroig *et al*., 2010). Functional analyses denoted that, although with some specificities, both *Sa* and *Ss* Elovl4a and Elovl4b are involved in VLC-SFA and VLC-PUFA biosynthesis, being able to elongate a range of substrates up to C_{34} VLC-SFA and VLC-PUFA. Moreover, neural tissues are the major site of *elovl4* expression, with brain and eye exhibiting the highest *elovl4a* and *elovl4b* expression levels, respectively. Therefore, these are likely the main tissues of VLC-FA biosynthesis and accumulation, which highlights the importance of these compounds for crucial

physiological processes, such as vision and brain function, particularly during early fish development.

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CAPÍTULO 4:

Expression of genes related to long-chain (C_{20-24}) and very long-chain ($>C_{24}$) fatty acid biosynthesis in gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*) larvae: Investigating early ontogeny and nutritional regulation

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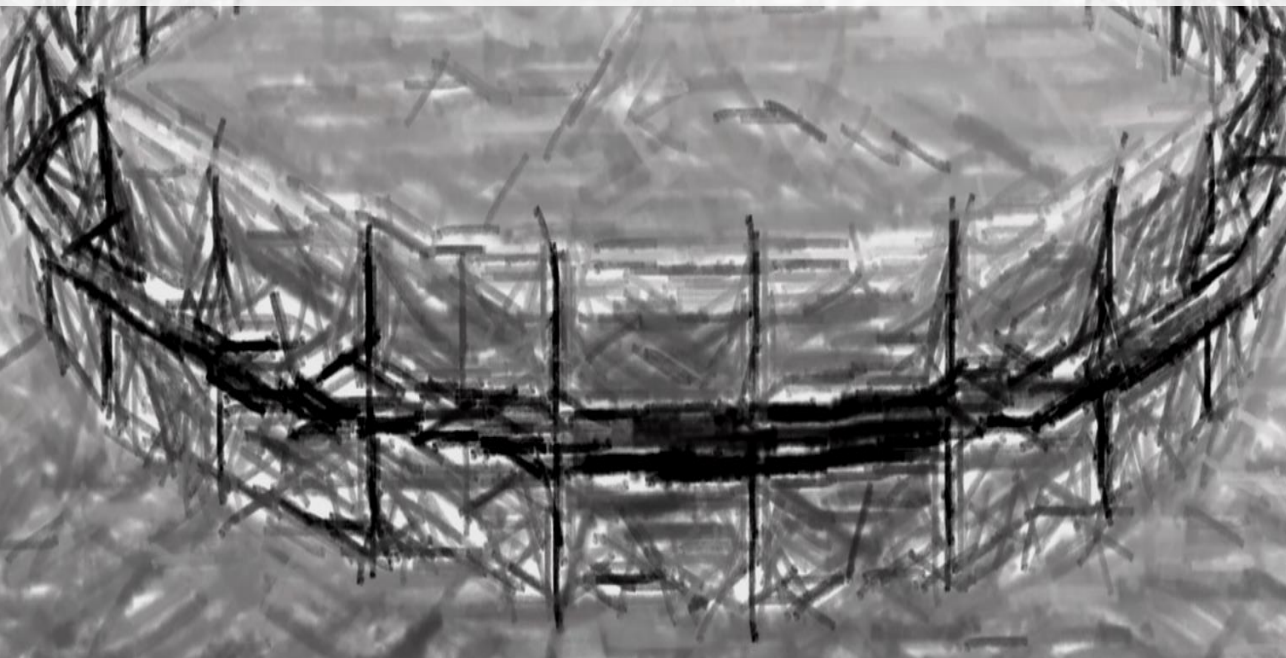

Abstract

Long-chain polyunsaturated fatty acids (LC-PUFA) have been extensively studied in aquaculture due to their importance for survival and development in teleosts. However, very long-chain polyunsaturated fatty acids (VLC-PUFA) have been practically unexplored within the aquaculture scenario. VLC-PUFA, although always present in small amounts, can be pivotal for the correct development and function of tissues such as retina, brain and gonads of vertebrates including fish. This study aimed at determining the temporal expression patterns of genes involved in the biosynthesis of VLC-PUFA (*elovl4a*, *elovl4b*) and their precursors, LC-PUFA (*fads2*, *elovl5*), during the early ontogeny of *Solea senegalensis* and *Sparus aurata*. Furthermore, we investigated the nutritional regulation of these genes in early life-cycle stages of fish fed low and high LC-PUFA diets consisting of non-enriched and enriched live preys (*Brachionus plicatilis* and *Artemia franciscana*), respectively. The effect of dietary LC-PUFA on growth and fatty acid composition was also examined. The results obtained during early development reveal that all genes studied are expressed before the hatching stage. There is a consistency between the timing at which retinogenesis occurs in both species and an increase of the expression of the two *elovl4* responsible for the synthesis of VLC-PUFA. The results obtained in nutritional assays for both species suggest that the expression of the two isoforms of *elovl4* (isoform *a* in early larvae, and *b* in late larvae) can be regulated

positively according to the dietary content of LC-PUFA in early stages, which could activate the VLC-PUFA biosynthesis even during short-term feeding periods (seven days). The body part analysis in late larvae of both species revealed that both isoforms of *elovl4* are expressed preferentially in the head. This can be associated to their highest presence in the neural and visual tissues.

Keywords: *Solea senegalensis*; *Sparus aurata*; Marine larvae; Very long-chain polyunsaturated fatty acid; Elovl4.

4.1. Introduction

One of the yet unresolved bottlenecks of intensive farming of many marine fish species is the lack of understanding of nutritional requirements of early life-cycle stages, where fish undergo dramatic morphological and physiological changes that determine their viability in later stages (Hamre *et al*., 2013; Izquierdo *et al*., 2015). Some lipids have been recognized as important nutritional components determining larval growth and development, and ultimately, survival (Izquierdo *et al*., 2000; Jobling, 2016; Tocher, 2010). Among them, long-chain (C_{20-24}) polyunsaturated fatty acids (LC-PUFA) are physiologically important nutrients for visual and cognitive development during early ontogeny, important for normal growth, as well as for tissue repair during injury (Bell and Tocher, 1989; Bell *et al*., 1995; Hamre *et al*., 2013; Jobling, 2016). Consequently, LC-PUFA such as arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), are important nutrients for the normal growth and development of marine fish larvae, where neural tissues accumulating these compounds are rapidly forming.

Polyunsaturated fatty acids (PUFA) of 18 carbons, namely α-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6), are dietary essential nutrients for all vertebrates since they cannot be synthesized *de novo* (Castro *et al*., 2016; Skov *et al*., 2013). C_{18} PUFA do not play vital roles in vertebrates *per se* but are the precursors

of the physiologically active C_{20-24} LC-PUFA (Monroig *et al*., 2018). Fish species vary in their capacity to convert C_{18} PUFA into C_{20-24} LC-PUFA, depending on the repertoire of fatty acyl elongase (*elovl*) and desaturase (*fads*) genes and the substrate specificities of their protein products (i.e. enzymes) (Li *et al*., 2010; Oboh *et al*., 2017a). Fads, with desaturase species-specific activity, and Elovl5 are limiting enzymes considered key in marine teleost LC-PUFA biosynthesis (Monroig *et al*., 2018).

Fads introduce double bonds into specific position within the fatty acyl chain, and desaturases with Δ4, Δ5, Δ6 and Δ8 activities have been demonstrated to play major roles in LC-PUFA biosynthesis in fish (Castro *et al*., 2016; Monroig *et al.*, 2018). Despite such a remarkable functional diversity, virtually all Fads-like desaturases from fish are *fads2* orthologs, with the exception of basal teleosts such as the Japanese eel (*Anguilla japonica*) possessing a Fads1 (Δ5 desaturase) (Lopes-Marques *et al*., 2018). Among Elovl, enzymes that catalyze the first reaction (condensation) of the elongation pathway resulting in the addition of two carbons to the preexisting fatty acyl chain (Castro *et al*., 2016; Monroig *et al*., 2018), three types, namely Elovl2, Elovl4 and Elovl5, participate in PUFA elongation (Jakobsson *et al*., 2006). All teleostean fish possess at least one Elovl5 and two Elovl4, the latter termed as "Elovl4a" and "Elovl4b" based on the nomenclature of the zebrafish *Danio rerio* orthologs (Monroig *et al*., 2010). However, Elovl2 has been lost during

evolution of teleosts and this elongase is absent from many marine farmed species (Castro *et al*., 2016; Monroig *et al*., 2018).

Unlike C_{20-24} LC-PUFA, very long-chain ($>C_{24}$) PUFA (VLC-PUFA) have been barely investigated in fish, despite the key roles that these compounds have in vision, brain function, skin permeability and reproduction of mammals (Agbaga *et al*., 2008; Aldahmesh *et al*., 2011; Furland *et al*., 2007; Mandal *et al*., 2004; Poulos, 1995). Investigations of VLC-PUFA in fish have been mostly restricted to the characterization of Elovl4 enzymes involved in their biosynthesis (Monroig *et al*., 2010; Oboh *et al*., 2017b; Carmona-Antoñanzas *et al*., 2011; Jin *et al*., 2017b; Kabeya *et al*., 2015). Fish Elovl4 proteins can actively elongate a range of PUFA substrates producing in some instances VLC-PUFA up to 36 carbons (Monroig *et al*., 2018). It is interesting to note that some fish Elovl4 enzymes have the ability to elongate 22:5n-3 to 24:5n-3 (Monroig *et al*., 2011, 2012), suggesting that these enzymes, in addition to their major role in VLC-PUFA biosynthesis, can contribute to the LC-PUFA biosynthesis thus denoting shared roles in both pathways (Jin *et al*., 2017a). Such enzymatic ability by fish Elovl4 has been also hypothesized to partly compensate for the above mentioned absence of Elovl2 in many fish species (Monroig *et al*., 2011, 2018). However, it is unknown which impacts might exist when supply of LC-PUFA, precursors of fish VLC-PUFA, is restricted in fish feeds. In the context of fish farming, dietary restriction of LC-PUFA is becoming an

extended trend due to the scarce availability of marine (i.e. LC-PUFA rich) ingredients such as fish meal and fish oil (Shepherd *et al*., 2017; Ytrestøyl *et al*., 2015), and therefore it is important to investigate the molecular mechanisms underlying the biosynthetic pathways of VLC-PUFA, especially during early developmental stages undergoing central physiological processes in which these compounds are involved (Monroig *et al*., 2010). Moreover, it is interesting to understand how their expression patterns can be regulated through the diet at the onset of exogenous feeding with live preys varying in their contents of LC-PUFA.

This study aimed at determining the temporal expression patterns of genes involved in the biosynthesis of VLC-PUFA (*elovl4a*, *elovl4b*) and their precursors, LC-PUFA (*fads2*, *elovl5*), during the early ontogeny of *Solea senegalensis* and *Sparus aurata*. These are the sole genes that participate in the biosynthetic pathways of LC and VLC-PUFA. Furthermore, we investigated the nutritional regulation of these genes in early life-cycle stages of fish fed low and high LC-PUFA diets consisting of non-enriched and enriched live preys, respectively. The species chosen as models in this study, namely *S. senegalensis* and *S. aurata*, are representative of marine fish species with different LC-PUFA biosynthesis strategies, particularly with regards to Fads2 functionality (Castro *et al*., 2016; Monroig *et al*., 2018). *S. aurata* possesses one sole Fads2 enzyme with Δ6, and to a lesser extent, Δ5 desaturase activities (Seiliez *et al*., 2003; Zheng *et al*., 2004). Moreover, *S. senegalensis* possess

a Fads2 with Δ4 activity (Morais *et al*., 2012) enabling the culture of its larval stages on diets (non-enriched live preys) containing negligible DHA and low EPA levels without obvious detrimental effects on growth and survival (Morais *et al*., 2012; Villalta *et al*., 2005). These enzymatic differences in LC-PUFA biosynthesis, along to other characteristics, as their specific larval development, and the different feeding habits, i.e. pelagic or benthonic, are of special interest to study the nutritional regulation of *elovl4* genes in different marine teleosts fed diets with a similar LC-PUFA content.

4.2. Materials and methods

4.2.1 Larval culture

Fertilized eggs of *S. senegalensis* and *S. aurata* were obtained from naturally spawning captive broodstocks from Stolt Sea Farm S.A. (A Coruña, Spain) and *Instituto Español de Oceanografía* (IEO) (Murcia, Spain), and hatched at 18 °C in filtered seawater with continuous recirculation at a density of ~400 eggs l^{-1}. Once hatched, larvae were reared in a closed system in 11-litre aquaria at an initial density of 100 larvae l^{-1}, temperature of 18-19 °C, photoperiod 12L:12D, and salinity of 37.5 ± 0.5 g l^{-1}. From the start of exogenous feeding at 4 days after hatching (dah) until 8 dah, larvae of both species were fed rotifers three times daily (*Brachionus plicatilis* fed *Tetraselmis* sp. at ~9 x 10^5 cells ml^{-1}) at a density of 5-10 rotifers ml^{-1}. For

nutritional regulation experiments, different diets were tested depending on early (16 dah) or late (40 dah) larvae.

4.2.2. Larval ontogeny

In order to study the temporal expression patterns of genes involved in the biosynthesis of LC- and VLC-PUFA (*elovl5*, *fads2*, *elovl4a* and *elovl4b*) during the early ontogeny of *S. senegalensis* and *S. aurata*, triplicate pools (~100 mg) of fertilized eggs, newly hatched larvae and larvae up to 7 dah were collected daily. Samples were immediately frozen and kept at -80 °C until further analysis.

4.2.3. Nutritional regulation

4.2.3.1. Experiment 1: Nutritional regulation in early larvae

A first experiment consisted of 9 dah larvae that were fed three times daily with rotifers (*Brachionus plicatilis*), which were obtained from cultures maintained at the facilities of IATS, enriched with Larviva Multigain (BioMar Iberia S.A., Palencia, Spain) with a proximate composition indicated by the supplier of 14 % crude protein, 43 % crude fats, 2.6 % crude fiber and 7.7 % crude ash (Rot E) or non-enriched (i.e., grown on the basal *Tetraselmis* sp. diet) (Rot NE) during 7 days in triplicate 11 l aquaria. Rot E were enriched according to the "short-term

enrichment" protocol (Dhert *et al*., 2001) in 3 l cylindro-conical flasks during 3 h at 28 °C with aeration, at a density of 300-350 individuals ml^{-1} in 30 g l^{-1} salinity diluted seawater. Prey density began at 10 rotifers ml^{-1} and was increased with larval age up to 15 rotifers ml^{-1} three times daily.

4.2.3.2. Experiment 2: Nutritional regulation in late larvae

In a second experiment, *S. senegalensis* and *S. aurata* larvae (25 dah) were reared in a closed recirculation system in triplicate 20 l aquaria at 25 larvae per aquaria and fed *Artemia franciscana* metanauplii, obtained from cysts with a proximate composition indicated by the distributor (INVE Aquaculture, NV., Dendermonde, Belgium) of 54 % crude protein, 11 % crude fats and 8 % crude ash, either enriched with Larviva Multigain (Art E) or bakery yeast (*Saccharomyces cerevisiae*) (Art NE) during 15 days. Enrichment was carried out in 3 l cylindro-conical flasks for 24 h at a density of 150-200 nauplii ml^{-1} in seawater at 28 °C and with strong aeration. Enrichment diets were supplied at 0.6 g l^{-1}, which were previously dispersed/homogenized in a known sea water volume using a stirrer. Prey density began at 5 nauplii ml^{-1} and was increased with larval age up to 15 nauplii ml^{-1} three times daily.

Fish samples (Experiments 1 and 2) were collected, weighed, measured and immediately frozen and kept at -80 °C until further analysis. For Experiment 2, due

to their larger size, head, viscera and muscle body compartment were dissected and analyzed separately.

4.2.3.3. Larval growth

After the larval feeding trials, samples of 16 dah early larvae (Experiment 1) and 40 dah late larvae (Experiment 2) were collected, and their total lengths (TL) and weights measured. Larval TL was measured with the digital image processing software ImageJ (Rueden *et al*., 2017). Late larvae were measured manually under a binocular microscope Leica MZ6 coupled to Transmitted-Light Base TO ST (MDG 28) (Leica Microsistemas S.L.U., Barcelona, Spain) with an ocular micrometer. Wet weight (WW) was recorded using a Mettler Toledo XS105 semi-micro balance (Mettler-Toledo S.A.E., Barcelona, Spain), as a pool of five animals for early larvae from Experiment 1, and individually for late larvae from Experiment 2. In all cases, at least 15 fish were used. Specific growth rate (SGR) was calculated as SGR = log (TL_f)-log (TL_i)/T (experimental time) * 100 (Lugert *et al*., 2016). Fulton's condition factor (K) of each late larvae was calculated as K = *WW* (g) / [*TL* (cm)]^3 x 100 (Froese, 2006). K was not calculated for early larval pools due to the impossibility of weighing each larva individually.

4.2.4. Fatty acid analysis

Total lipids of experimental diets (Rot E, Rot NE, Art E, and Art NE), and body compartments from larvae of Experiment 2, were extracted with chloroform/methanol (2:1, v/v) according to Folch *et al*. (1957) and quantified gravimetrically after evaporation of the solvent under nitrogen flow, followed by vacuum desiccation overnight. Total lipids were resuspended at 10 mg ml^{-1} in chloroform/methanol (2:1) containing 0.01 % (w/v) butylhydroxytoluene (BHT). Then, 100 µl of total lipids were subjected to an acid-catalyzed transesterification (Christie, 1982). Fatty acid methyl esters (FAME) were subsequently extracted using hexane/diethyl ether (1:1, v/v), and purified by TLC (Silica gel 60, VWR, Barcelona, Spain) as previously described (Christie, 1982). In the case of individual early larvae (Experiment 1), due to the small amount of sample, fatty acid (FA) profiles were obtained through an adapted direct transmethylation method (Garrido *et al*., 2016), and total lipid values are not available.

FA composition was determined using a Thermo Scientific TRACE GC Ultra gas chromatograph (Thermo Fisher Scientific, Madrid, Spain), equipped with a fused silica 30 m × 0.25 mm open tubular column (Tracer, TRB-WAX, film thickness: 0.25 µm, Teknokroma, Barcelona, Spain). Injections of 1 µl samples were carried out on-column, using helium as carrier gas (1.5 ml min^{-1} constant flow), and a thermal

gradient from 50 (injection temperature) to 220 °C, and reported as % of total fatty acids. Methyl esters were identified by comparison with known standards.

4.2.5. RNA extraction and real time quantitative PCR (qPCR)

Total RNA was isolated from three pools of whole fertilized eggs and larvae at various stages of development (0 to 7, and 16 dah), using Maxwell 16 LEV simplyRNA Tissue Kit (Promega Biotech Ibérica S.L., Madrid, Spain) following the manufacturer's instructions. From Experiment 2, head, viscera and muscle body compartments of late larvae (40 dah) were differentiated and processed separately. RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop ND-2000C, Thermo Fisher Scientific, Barcelona, Spain). Two micrograms of total RNA per sample was reverse transcribed into cDNA using the M-MLV reverse transcriptase first strand cDNA synthesis kit (Promega Biotech Ibérica S.L., Madrid, Spain) following manufacturer's instructions, using a mixture (3:1, mol/mol) of random primers and anchored oligo $(dT)_{15}$ primer (Promega Biotech Ibérica S.L., Madrid, Spain). Expression of fatty acyl desaturase (*fads2*) and elongases (*elovl5*, *elovl4a* and *elovl4b*) was quantified by qPCR using primers shown in Table 4.1. Primers were designed using Primer3 software (http://primer3.sourceforge.net) (Rozen and Skaletsky, 2000). The amplification efficiency of the primer pairs was assessed by serial dilutions of

standard solutions of the studied genes with known copy numbers that helped to build a standard curve, which also allowed the conversion of threshold cycle (Ct) values to copy numbers. Amplifications were carried out in technical duplicates on a qPCR thermocycler (CFX Connect Real-Time System, Bio-Rad Laboratories S.A., Madrid, Spain) in reactions with a final volume of 20 µl, containing 5 µl diluted (1/20) cDNA problem samples for all genes, except for *S. senegalensis β-actin* (*actb*) gene (1/200), 0.5 µl of each primer and 4 µl Master Mix qPCR No-ROX PyroTaq EvaGreen 5x (CMB-Bioline, Madrid, Spain). All runs included a systematic negative control consisting of a non-template control (NTC). The qPCR program consisted of an initial activation step at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, elongation at 72 °C for 15 s, and a final melt curve of 0.5 °C increments from 60 °C to 90 °C, enabling confirmation of the amplification of a single product in each reaction. Three potential housekeeping genes (*β-actin, elongation factor 1α and 18s rRNA*) were tested. Finally, next to check its stability using the Genorm software (Vandesompele *et al*., 2002), *β-actin* was used for normalization of the candidate gene expression. Gene expression results are given as mean normalized values (± SD) corresponding to the ratio between copynumbers of fatty acyl desaturase (*fads2*) and fatty acyl elongases (*elovl5*, *elovl4a* and *elovl4b*) transcripts and copy numbers of the reference gene *β-actin* (*actb*).

Table 4.1. Primers used for real-time quantitative PCR (qPCR) of *Sparus aurata* and *Solea senegalensis* genes. Sequences of the primer pairs used (Forward: F; Reverse: R), annealing temperatures (Ta) of the primer pairs, size of the fragments produced, and accession number of the sequences used for the primer design are shown**.**

Sparus aurata					
Transcript	**Primer**	**Primer sequence**	**Ta**	**Fragment**	**Accession No**
elovl4a	F	5'-GCCCAAGTACATGAAGAACAGAG-3'	60 °C	169 bp	MK610320
	R	3'-GGGGTCGTCTGAGTAGTCCA-5'			
elovl4b	F	5'-GTCAAGTACTCCAACGATGTCAA-3'	60 °C	247 bp	MK610321
	R	3'-TGAGCACATGGATGGAAGAG-5'			
elovl5	F	5'-TCGTCCACGTCGTGATGTAT-3'	60 °C	152 bp	Q68YU3
	R	3'-ACATGGCCATATGACTGCAA-5'			
fads2	F	5'-CACTCAGCCAGTCGAGTACG-3'	60 °C	199 bp	GQ162822
	R	3'-ACAGCACAGGTAGCGAAGGT-5'			
actb	F	5'-TGCGTGACATCAAGGAGAAG-3'	60 °C	190 bp	X89920
	R	3'-CAGGACTCCATACCGAGGAA-5'			
Solea senegalensis					
Transcript	**Primer**	**Primer sequence**	**Ta**	**Fragment**	**Accession No**
elovl4a	F	5'-AGGTGAGGTAGGGCCTTGTT-3'	60 °C	220 bp	MN164537
	R	3'-TGAAAACAGCCACCTTAGGC-5'			
elovl4b	F	5'-CCTCTGCCTTGTCCAGTTTC-3'	60 °C	175 bp	MN164625
	R	3'-CAATTTGATGCCCAGTTCCT-5'			
elovl5	F	5'-CAAGTACATGCAGCACAGGC-3'	60 °C	116 bp	JN793448
	R	3'-GCCACACAGCACTAACAAGC-5'			
fads2	F	5'-GTTCGTGTGGGTGACTCAGA-3'	60 °C	121 bp	JN673546
	R	3'-GTCGTTGAAGGAGGACTGCT-5'			
actb	F	5'-ACAATGAGCTGAGAGTCGCC-3'	60 °C	132 bp	DQ485686
	R	3'-CAACATACATGGCGGGGGTA-5			

4.2.6. Statistical analysis

For each species, data from gene expression on different stages along larval development (0-7 dah) and different body compartments (viscera, muscle and head) in late larvae (Experiment 2) were checked for homogeneity of variances using Levene's test and analyzed by one-way analysis of variance (ANOVA) ($P \leq 0.05$) followed by Tukey HSD *post-hoc* test. To compare the effects of the two experimental diets tested in Experiments 1 and 2, WW, TL, K, FA and gene expression data were checked for homogeneity of variances using Levene's test and then analyzed by an independent sample t-Student test, at significance levels of $P \leq 0.05$, except where noted otherwise. The statistical software SPSS 24.0 (SPSS Inc., Chicago, USA) was used to analyze the data.

4.3. Results

4.3.1. Temporal expression of fads2, elovl5, elovl4a and elovl4b during early development of S. aurata and S. senegalensis

The results of the temporal expression of *fads2*, *elovl5*, *elovl4a* and *elovl4b* revealed differences in the expression patterns for *S. aurata* and *S. senegalensis*. In both species, the results showed that all the candidate genes were expressed before

hatching stage, with transcripts detected throughout the entire developmental time frame studied (**Figures 4.1** and **4.2**).

For *S. aurata*, expression of *fads2*, *elovl5* and *elovl4a* showed a trend to increase until 5 dah (**Figures 4.1 A**, **B**, **C**). *Elovl4b* showed an expression pattern similar to the other genes, although the highest expression was detected at 4 dah, point after which there was a decrease (**Figure 4.1 D**). The lowest expression values shown in eggs for all the genes studied in comparison to post-hatching stages (1-7 dah) denoted a low transcriptional activity during early embryogenesis. For *S. senegalensis*, *fads2* showed an expression pattern characterized by the existence of two periods of high transcriptional activity. The first peak, at 1-2 dah, is consistent with those of *elovl4a*, *elovl4b*. Subsequently, *fads2* expression decreased and then increased showing a second peak at 5-6 dah (**Figure 4.2 A**). *Elovl5* showed its highest expression in eggs (**Figure 4.2 B**). After hatching, *S. senegalensis* presented a rapid increase in expression values for both *elovl4a* and *elovl4b*, showing a peak at 2 dah. Later, expression values decreased to remain relatively stable until the end of the period studied (**Figures 4.2 C**, **D**).

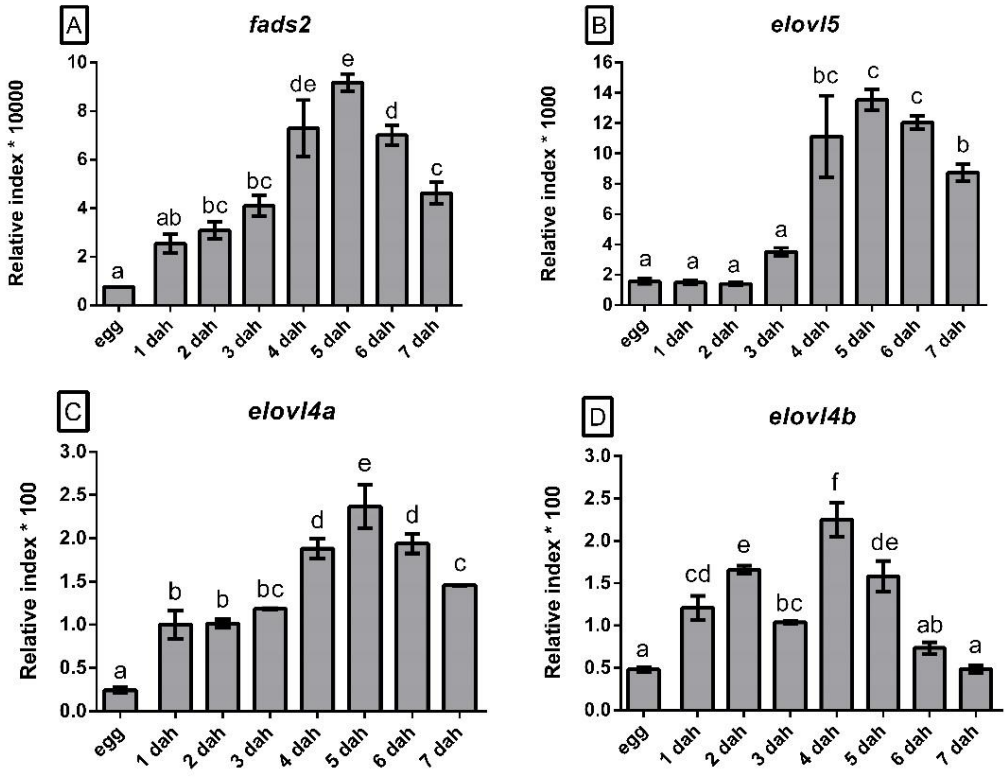

Figure 4.1. Expression pattern of *S. aurata* fatty acyl desaturase (*fads2*, **A**) and elongases (*elovl5*, **B**; *elovl4a*, **C**; *elovl4b*, **D**) genes during early ontogenetic development, determined by qPCR in whole eggs and larvae from 1 to 7 days after hatching (dah). The results shown as relative index, are β-actin normalized values (gene copy number/*β-actin* copy number) corresponding to the mean and standard deviation as error bars (n = 3). Different letters above the columns show significant differences (ANOVA and Tukey test, $P \leq 0.05$) among time points for each gene.

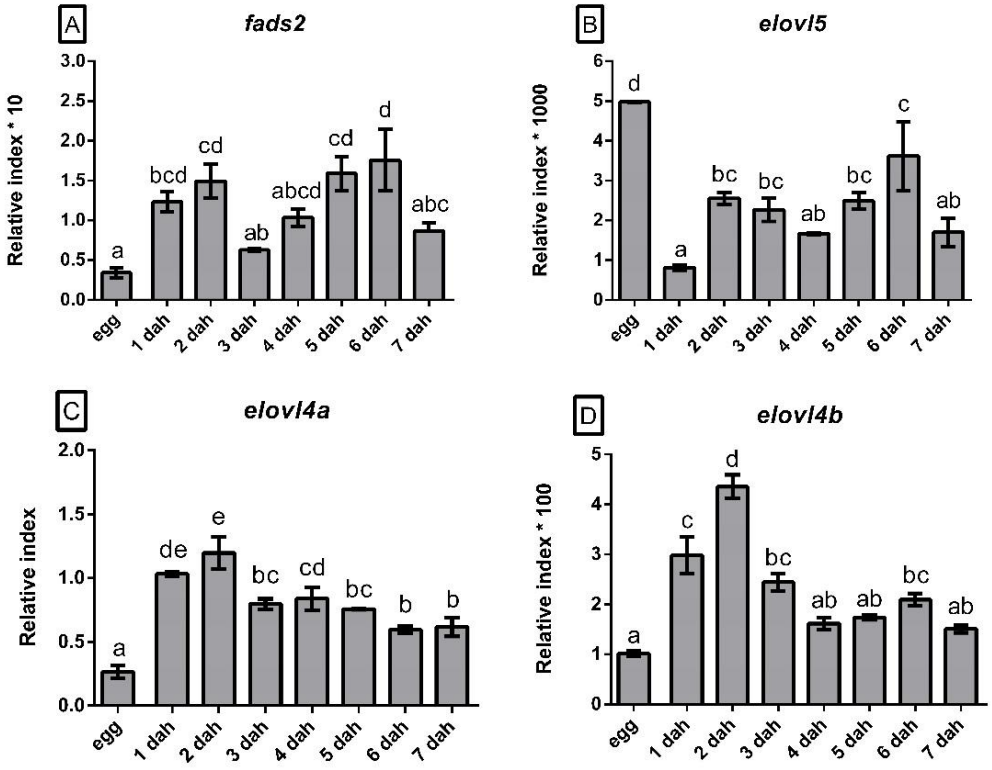

Figure 4.2. Expression pattern of *S. senegalensis* fatty acyl desaturase (*fads2*, **A**) and elongases (*elovl5*, **B**; *elovl4a*, **C**; *elovl4b*, **D**) genes during early ontogenetic development, determined by qPCR in whole eggs and larvae from 1 to 7 days after hatching (dah). The results shown as relative index, are β-actin normalized values (gene copy number/*β-actin* copy number) corresponding to the mean and standard deviation as error bars (n = 3). Different letters above the columns show significant differences (ANOVA and Tukey test, $P \leq 0.05$) among time points for each gene.

4.3.2. Nutritional regulation experiments

4.3.2.1. Larval growth

Results obtained for larval growth are shown in Table 4.2. Generally, early and late larvae (Experiments 1 and 2) fed enriched diets (Rot E and Art E) presented higher growth performance at the end of the experimental periods, as fish show a higher TL and WW compared to non-enriched diets (Rot NE and Art NE).

In Experiment 1, *S. aurata* early larvae fed the Rot E diet presented higher WW than early larvae fed Rot NE diet. For *S. senegalensis*, early larvae fed Rot E diet presented higher TL than early larvae fed the Rot NE diet (Table 4.2).

In Experiment 2, *S. aurata* late larvae fed the Art E diet presented higher TL and WW than early larvae fed the Art NE diet. For *S. senegalensis*, late larvae fed the Art E diet presented higher WW than late larvae fed the Art NE diet. No significant differences were found between dietary regimes in the Fulton's K condition factor values for late larvae of both species (Table 4.2).

Table 4.2. Growth of *S. aurata* and *S. senegalensis* larvae fed with different live preys: rotifer enriched (Rot E) *vs* non-enriched (Rot NE), and *Artemia* metanauplii enriched (Art E) *vs* non-enriched (Art NE). Length, weight, specific growth rate (SGR) and Fulton's K condition factor are presented as mean ± SD (n = 15). The symbol " * " shows significant differences (t-Student, $P \leq 0.05$) between the dietary regimes.

	S. aurata		***S. senegalensis***	
Diet	**Rot E**	**Rot NE**	**Rot E**	**Rot NE**
Total Length (mm)	4.33 ± 0.09	4.06 ± 0.18	4.23 ± 0.05 *	4.01 ± 0.07
Wet Weight (mg)	1.07 ± 0.17 *	0.55 ± 0.04	0.62 ± 0.07	0.58 ± 0.03
SGR	0.54 %	0.35 %	0.80 %	0.41 %
Diet	**Art E**	**Art NE**	**Art E**	**Art NE**
Total Length (mm)	21.17 ± 2.10*	17.37 ± 2.30	27.83 ± 2.45	24.33 ± 1.93
Wet Weight (mg)	94.83 ± 2.77 *	52.54 ± 2.23	171.20 ± 4.91*	106.10 ± 2.47
SGR	1.27 %	0.70 %	0.98 %	0.64 %
Fulton's K	0.98 ± 0.01	1.00 ± 0.05	0.69 ± 0.03	0.67 ± 0.03

4.3.2.2. Fatty acid composition

Effects of dietary LC-PUFA during different windows of development (early larvae and late larvae) of *S. aurata* and *S. senegalensis* were investigated using enriched and non-enriched live preys (rotifers and *Artemia*). For Experiment 1, the Rot E diet consisted of rotifers enriched with Larviva Multigain containing high levels of n-6 docosapentaenoic acid (n-6 DPA; 22:5n-6) and DHA, while the Rot NE diet, i.e. rotifers grown on *Tetraselmis* sp., contained high levels of ALA, stearidonic acid (SDA; 18:4n-3) and eicosatetraenoic acid (ETA; 20:4n-3) (Table 4.3). For Experiment 2, the Art E diet had high levels of ARA, EPA, n-6 DPA and DHA, with the Art NE diet being characterized by high levels of ALA and LA (Table 4.3).

Table 4.3. Selected fatty acids (% total fatty acids) of the experimental diets: enriched (E) *vs* non-enriched (NE) live preys. Results are expressed as mean ± SD (n = 3). The symbol " * " indicates significant differences in fatty acid content of the two diets for each live prey (t-Student, $P \leq 0.05$).

Fatty acid	*Rotifers*		*Artemia* metanauplii	
	E	NE	E	NE
18:2n-6 (linoleic acid**)**	3.06 ± 0.37	3.78 ± 0.32	4.14 ± 0.02*	5.52 ± 0.01
18:3n-6 (γ-linolenic acid**)**	0.14 ± 0.04	0.21 ± 0.02	-	-
20:2n-6 (eicosadienoic acid)	0.07 ± 0.00	0.13 ± 0.02	0.17 ± 0.01	0.25 ± 0.00
20:3n-6 (dihomo-γ-linolenic acid**)**	0.18 ± 0.06	0.14 ± 0.01	-	-
20:4n-6 (arachidonic acid**)**	0.89 ± 0.29	0.96 ± 0.03	2.73 ± 0.03*	1.14 ± 0.00
22:2n-6 (docosadienoic acid)	0.11 ± 0.00	0.14 ± 0.00	-	-
22:4n-6 (adrenic acid**)**	0.08 ± 0.00	0.11 ± 0.04	0.10 ± 0.01	-
22:5n-6 (n-6 docosapentaenoic acid**)**	4.08 ± 2.12*	0.40 ± 0.28	7.79 ± 0.02*	-
Total n-6 PUFA	8.61 ± 2.88	5.87 ± 0.73	14.94 ± 1.43*	6.91 ± 1.26
18 3n-3 (α-linolenic acid**)**	2.79 ± 0.32*	10.46 ± 0.68	13.90 ± 0.17*	23.81 ± 0.05
18 4n-3 (stearidonic acid**)**	1.82 ± 0.34*	4.38 ± 0.17	1.77 ± 0.04*	3.56 ± 0.05
20 3n-3 (docosatrienoic acid**)**	0.18 ± 0.02*	0.44 ± 0.03	0.48 ± 0.01*	0.76 ± 0.01
20:4n-3 (eicosatetraenoic acid**)**	1.85 ± 0.34*	3.67 ± 0.14	0.63 ± 0.01	0.69 ± 0.01
20:5n-3 (eicosapentaenoic acid**)**	1.30 ± 0.41*	2.72 ± 0.06	5.33 ± 0.05*	2.91 ± 0.01
22:3n-3 (docosatrienoic acid)	0.13 ± 0.02	0.23 ± 0.04	-	0.13 ± 0.01
22:5n-3 (n-3 docosapentaenoic acid**)**	0.30 ± 0.12	0.40 ± 0.01	0.33 ± 0.00	-
22:6n-3 (docosahexaenoic acid)	7.65 ± 0.55*	2.51 ± 0.03	17.82 ± 0.09*	-
Total n-3 PUFA	15.87 ± 2.10*	24.59 ± 1.13	40.25 ± 2.40*	31.96 ± 2.86
Total unsaturates	37.44 ± 7.24	52.90 ± 4.87	73.78 ± 1.05	75.78 ± 1.48
Total saturates	35.37 ± 3.81	24.88 ± 3.56	23.00 ± 1.99	19.50 ± 1.40
Total MUFA	12.34 ± 2.06	21.09 ± 2.77	18.09 ± 1.20*	36.02 ± 2.30
Total PUFA	25.10 ± 5.18	31.81 ± 2.10	41.87 ± 1.22*	39.76 ± 1.67
Total lipids (%)	15.22 ± 1.00	11.62 ± 0.60	21.60 ± 0.78*	13.77 ± 0.36

Totals include some components not shown. MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; (-): not detected; Total lipids (%): percentage of lipids with respect to the total dry weight of the sample analyzed.

Fatty acid analyses for Experiment 1 (early larvae) denoted that *S. aurata* and *S. senegalensis* larvae fed Rot E diet showed the highest content of DHA and n-6 DPA, while *S. aurata* and *S. senegalensis* larvae fed Rot NE diet had the highest content of LA, ALA, and EPA (Table 4.4).

For Experiment 2 (late larvae), both species fed Art E diet showed the highest content of PUFA in the body part analyzed (muscle). *S. aurata* larvae fed Art E diet showed the highest content of EPA, n-6 DPA and DHA, while those fed Art NE diet contained the highest LA and ALA (Table 4). *S. senegalensis* larvae fed Art E diet showed the highest content of ARA, n-6 DPA, EPA and DHA, while those fed Art NE diet contained the highest LA and ALA (Table 4.4).

Table 4.4. Selected fatty acids content (% total fatty acids) of *S. aurata* and *S. senegalensis* early larvae (fed enriched -Rot E- or non-enriched -Rot NE- rotifers) and late larvae (fed enriched -Art E- or non-enriched -Art NE- *Artemia* metanauplii) muscle. Results are expressed as mean ± SD (n = 3). The symbol " * " indicates significant differences in selected fatty acids between larvae fed the two corresponding diets (t-Student, $P \leq 0.05$).

Fatty acid	*S. aurata early larvae*		*S. senegalensis early larvae*		*S. aurata late larvae*		*S. senegalensis late larvae*	
	Rot E	**Rot NE**	**Rot E**	**Rot NE**	**Art E**	**Art NE**	**Art E**	**Art NE**
18:2n-6	3.35 ± 0.11*	4.03 ± 0.10	3.88 ± 0.11*	4.43 ± 0.11	3.17 ± 0.18*	4.90 ± 0.33	4.58 ± 0.08*	6.28 ± 0.06
18:3n-6	0.14 ± 0.04	0.15 ± 0.01	0.12 ± 0.01	0.15 ± 0.06	0.17 ± 0.02	0.15 ± 0.01	-	-
20:2n-6	0.22 ± 0.01	0.23 ± 0.01	0.44 ± 0.01	0.52 ± 0.03	0.10 ± 0.01*	0.16 ± 0.01	0.30 ± 0.02	0.38 ± 0.03
20:3n-6	0.46 ± 0.01	0.48 ± 0.03	0.38 ± 0.02	0.29 ± 0.04	-	-	-	-
20:4n-6	4.75 ± 0.12	4.66 ± 0.27	4.68 ± 0.06	4.13 ± 0.18	4.75 ± 0.27	4.61 ± 0.26	5.11 ± 0.03*	2.75 ± 0.04
22:4n-6	1.65 ± 0.05	1.74 ± 0.01	1.57 ± 0.14	1.82 ± 0.04	0.22 ± 0.03	0.15 ± 0.03	1.00 ± 0.06*	1.41 ± 0.03
22:5n-6	3.48 ± 0.24*	0.95 ± 0.05	4.43 ± 0.12*	1.32 ± 0.04	4.86 ± 0.36*	0.44 ± 0.04	3.23 ± 0.14*	0.24 ± 0.02
Total n-6 PUFA	14.04 ± 0.57*	12.25 ± 0.48	15.50 ± 0.46*	12.67 ± 0.49	13.26 ± 0.86*	10.41 ± 0.67	14.22 ± 0.33*	11.07 ± 0.17

18:3n-3	1.49 ± 0.25*	4.06 ± 0.52	1.73 ± 0.16*	4.34 ± 0.24	1.99 ± 0.07*	2.62 ± 0.10	6.87 ± 0.46*	10.30 ± 0.26
18:4n-3	0.84 ± 0.12	1.35 ± 0.15	0.88 ± 0.08*	1.71 ± 0.09	0.20 ± 0.02*	0.17 ± 0.01	0.66 ± 0.07	0.85 ± 0.04
20:3n-3	0.31 ± 0.09	0.53 ± 0.02	0.32 ± 0.04*	0.56 ± 0.04	0.12 ± 0.01*	0.18 ± 0.01	0.37 ± 0.12	0.52 ± 0.12
20:4n-3	2.86 ± 0.29	4.20 ± 0.20	2.68 ± 0.15*	3.61 ± 0.14	0.23 ± 0.01*	0.27 ± 0.01	0.54 ± 0.10*	0.92 ± 0.08
20:5n-3	4.38 ± 0.16*	6.18 ± 0.09	2.11 ± 0.03*	3.48 ± 0.06	9.08 ± 0.46*	7.43 ± 0.03	3.42 ± 0.16*	2.66 ± 0.12
22:4n-3	0.11 ± 0.01	0.17 ± 0.02	0.14 ± 0.01	0.18 ± 0.02	0.07 ± 0.02	0.06 ± 0.00	0.31 ± 0.02*	0.47 ± .020
22:5n-3	2.75 ± 0.11	3.34 ± 0.09	2.21 ± 0.10	2.27 ± 0.05	0.71 ± 0.05	0.79 ± 0.07	2.28 ± 0.08*	1.32 ± 0.07
22:6n-3	25.48 ± 1.16*	16.71 ± 1.15	25.09 ± 0.40*	18.85 ± 0.65	13.42 ± 1.58*	4.48 ± 0.36	10.43 ± 0.40*	3.10 ± 0.24
Total n-3 PUFA	38.22 ± 2.18	36.54 ± 2.14	35.16 ± 0.97	35.01 ± 1.28	24.11 ± 2.10*	17.59 ± 1.02	30.00 ± 1.45*	22.87 ± 0.98
Total unsaturates	68.95 ± 3.78	69.16 ± 3.70	68.56 ± 2.75	69.30 ± 2.56	59.99 ± 5.01*	54.43 ± 2.93	65.93 ± 2.49	63.42 ± 1.85
Total saturates	30.46 ± 0.86	30.42 ± 0.91	31.03 ± 1.28	30.11 ± 0.92	22.06 ± 0.90	22.99 ± 1.33	32.08 ± 1.71	29.23 ± 1.06
Total MUFA	15.91 ± 0.89*	19.64 ± 0.86	16.91 ± 0.95*	20.24 ± 0.71	21.05 ± 1.65	25.01 ± 1.01	25.83 ± 0.69*	30.95 ± 0.59
Total PUFA	53.04 ± 2.89*	49.52 ± 2.84	51.66 ± 1.80*	49.06 ± 1.85	37.44 ± 2.98*	28.06 ± 1.69	38.70 ± 1.74*	31.19 ± 1.12
Total lipids (%)	Not quantified	Not quantified	Not quantified	Not quantified	10.17 ± 0.94*	7.94 ± 0.92	13.54 ± 2.5	10.40 ± 1.97

Totals include some components not shown. MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; (-): not detected; Total lipids (%): percentage of lipids with respect to the total dry weight of the sample analyzed.

4.3.2.3. Gene expression

In Experiment 1, early larvae of both species showed an expression pattern characterized by an up-regulation of *elovl4a*, when fed enriched rotifers. However, only *S. aurata* larvae showed significant differences between different dietary treatments for *elovl4a* gene (**Figure 4.3 A**). Regarding to *S. senegalensis*, larvae did not show significant differences between diets, but it is important to note that P values ($P < 0.07$, **Figure 4.3 B**) close to the significance limit of 0.05 were obtained. No significant differences were found in the expressions of *fads2*, *elovl5*, and *elovl4b*

genes for *S. aurata* (**Figure 4.3 A**), or *S. senegalensis* (**Figure 4.3 B**) in response to diet (E-NE).

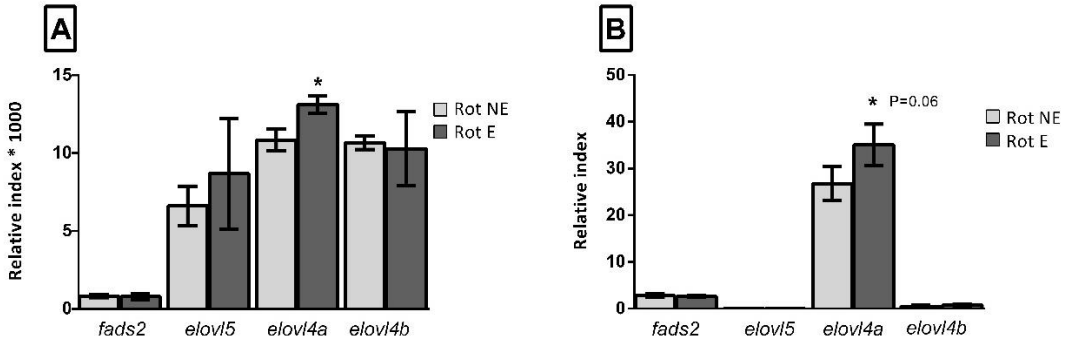

Figure 4.3. Expression pattern of *S. aurata* (**A**) and *S. senegalensis* (**B**) fatty acyl desaturase (*fads2*) and elongase (*elovl4a*, *elovl4b* and *elovl5*) genes in early larvae (16 days after hatching) fed rotifer diets: enriched (Rot E) and non-enriched (Rot NE). The results, shown as relative index, are β-actin normalized values (gene copy number / *β-actin* copy number) corresponding to the mean and standard deviation as error bars (n = 3). Different letters above the columns show significant differences (t-Student, $P \leq 0.05$, except where noted) between the diets, for each gene.

In Experiment 2, for *S. aurata* late larvae, no significant differences were observed in the dietary regulation of *fads2*, *elovl5*, *elovl4a* and *elovl4b*, as a consequence of different dietary LC-PUFA content (**Figure 4.4**). For *S. senegalensis* late larvae, differences were observed in the dietary regulation of *fads2*, whose expression was up-regulated in late larvae fed diet Art NE, i.e. low LC-PUFA diet. However, although no significant differences were observed in the expression of both isoforms of *elovl4* in response to dietary LC-PUFA, *elovl4b* ($P < 0.07$, **Figure 4.5 D**) appeared to be up-regulated in the head of *S. senegalensis* late larvae fed diet

Art E, denoting an opposite regulatory mechanism to that of *fads2* and *elovl5*, in response to dietary LC-PUFA (**Figure 4.5**).

The results of body fraction analysis (viscera, muscle and head) revealed significant differences in the expression patterns of all genes studied (*fads2*, *elovl5*, *elovl4a* and *elovl4b*). For *S. aurata*, *fads2* showed the highest expression levels in the head (**Figure 4.4 A**), whereas *elovl5* peaked in the viscera and head (**Figure 4.4 B**). Besides, the head showed the highest expression levels for *elovl4a* and *elovl4b* (**Figures 4.4 C, D**). For *S. senegalensis*, *fads2* and *elovl5* presented the highest expression levels in the visceral zone (**Figures 4.5 A, B**) and *elovl4a* and *elovl4b* in the head (**Figures 4.5 C, D**).

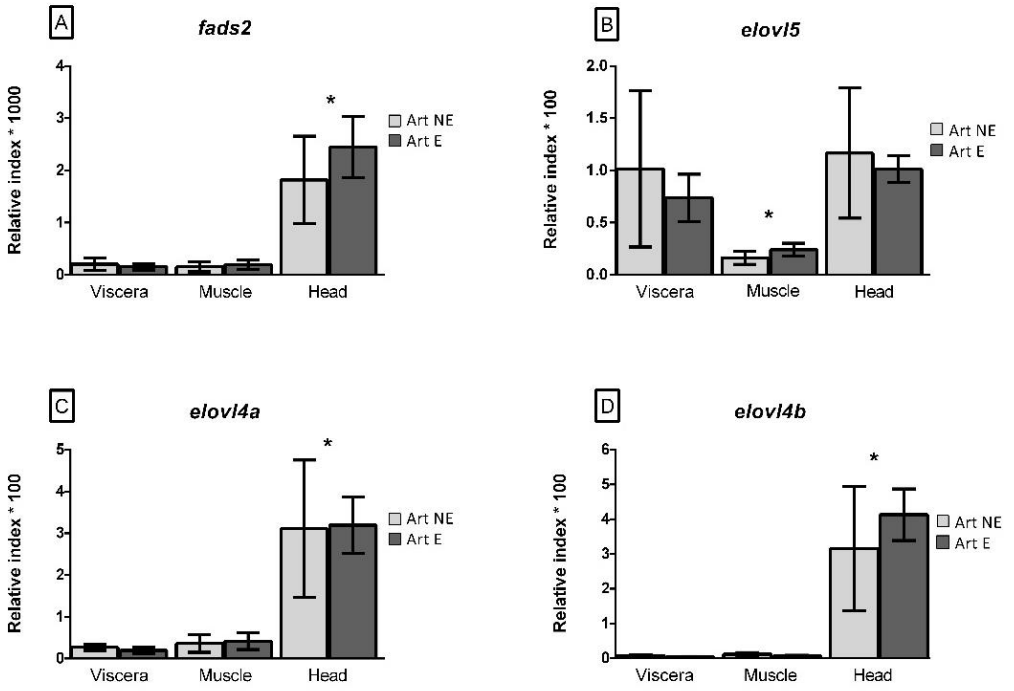

Figure 4.4. Expression pattern of *fads2* (**A**), *elovl5* (**B**), *elovl4a* (**C**) and *elovl4b* (**D**) in *S. aurata* late larvae (40 days after hatching) fed *Artemia* diets: enriched (Art E) and non-enriched (Art NE). The results, shown as relative index, are β-actin normalized values (gene copy number / *β-actin* copy number) corresponding to the mean and standard deviation as error bars (n = 3). The symbol " * " above the columns shows significant differences (one way-ANOVA, $P \leq 0.05$) among body compartments for both diets pooled.

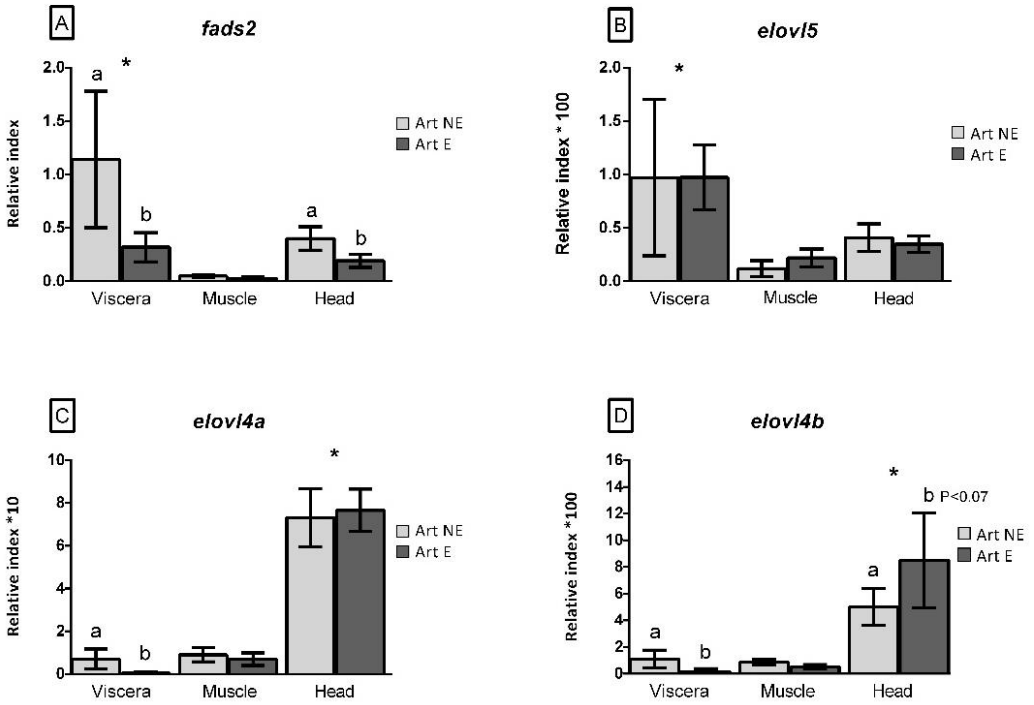

Figure 4.5. Expression pattern of *fads2* (**A**), *elovl5* (**B**), *elovl4a* (**C**) and *elovl4b* (**D**) in *S. senegalensis* late larvae (40 days after hatching) fed *Artemia* diets: enriched (Art E) and non-enriched (Art NE). The results shown as relative index, are β-actin normalized values (gene copy number / *β-actin* copy number) corresponding to the mean and standard deviation as error bars (n = 3). Different letters above the columns represent significant differences (t-Student, $P \leq 0.05$, except where noted) between diets, for each gene. The symbol " * " above the columns shows significant differences (one way-ANOVA, $P \leq 0.05$) among body compartments for both diets pooled.

4.5. Discussion

Several studies on commercially important fish species have emphasized the importance of VLC-PUFA in aquaculture (Carmona-Antoñanzas *et al*., 2011; Jin *et al*., 2017b; Monroig *et al*., 2012; Oboh *et al*., 2017b; Zhao *et al*., 2019). At present, the analysis of VLC-PUFA remains challenging due to the low presence of these compounds in tissues, their fragmentation during the chromatographic analysis and the lack of reference standards commercially available (Agbaga *et al*., 2010; Garlito *et al*., 2019). However, establishing the roles of Elovl4 in VLC-PUFA biosynthetic pathways and how their activity can be regulated through the diet has been identified central to understand the impacts that current feeding strategies, including the effects of a dietary reduction of VLC-PUFA precursors (i.e. LC-PUFA) can have on farmed fish. Physiological roles of Elovl4 products in vision and brain function make early development stages particularly vulnerable (Monroig *et al*., 2010), and this study aimed to investigate the metabolic and compositional responses of early life-cycle stages of *S. senegalensis* and *S. aurata* when fed diets with varying levels of LC-PUFA.

The results obtained in both species, for temporal expression of genes involved in the biosynthesis of LC-PUFA (*elovl5*, *fads2*) and VLC-PUFA (*elovl4a*, *elovl4b*) by qPCR, reveal the existence of inter- and intra-specific differences. On one hand, we observed a differential increase of the expression levels of the two *elovl4* genes in

both species. On the other hand, peaks of expression of *elovl4* genes differed between species but, in each case, these were consistent with timing at which the most relevant processes involved in retinogenesis occurs during larval development of *S. senegalensis* (early after hatching) (Bejarano-Escobar *et al*., 2010) and *S. aurata* (late after hatching) (Pavón-Muñoz *et al*., 2016).

Generally, fishes have a well differentiated three-layered retina (Pavón-Muñoz *et al*., 2016). During early stages of development, however, the vertebrate neuroretina consists of a neuroepithelium composed of undifferentiated retinal progenitor cells (Pavón-Muñoz *et al*., 2016; Turner and Cepko, 1987). Later, altricial fish larvae, experience a process of retinal maturation where tissue differentiation is carried out until the development of a mature retina (Pavón-Muñoz *et al*., 2016). During this process, where fish undergo dramatic morphological and physiological changes, it is important to have an optimal reserve of nutrients that allows to face the changes that occur during larval ontogenesis. LC-PUFA, especially DHA, is a major component of biological membranes, particularly of immune cells and neural tissue, being vital for visual and cognitive development during early ontogeny (Bell and Tocher, 1989; Bell *et al*., 1995). Moreover, there are different studies that relate *elovl4* disarranges and an inefficient level of their biosynthesis products with the development of visual disorders in vertebrates (Barabas *et al*., 2013; Maugeri *et al*., 2004), since VLC-PUFA, although in small amounts, are present in retina, associated

with the phosphatidylcholine from the outer membranes (Aveldaño and Sprecher, 1987). For this reason, we suggest that the synchrony between the timing at which retinogenesis occurs in both species and an increased expression of the two *elovl4* genes could highlight the importance of VLC-PUFA for the correct development of vision during early larval development of fish. There is a temporal decoupling in the expression of both *S. aurata elovl4* isoforms, since *elovl4a* showed a maximum activity at 5 dah, while *elovl4b* exhibited an advanced peak at 4 dah. This temporal decoupling could be indicative of differences existing at level of substrate specificity and/or tissue localization of both isoforms. Although the functional characterization of *S. aurata* and *S. senegalensis* Elovl4a and Elovl4b have not been yet published, the function of Elovl4 enzymes has been characterized in aquaculture species such as *Siganus canaliculatus*, *Clarias gariepinus*, *Salmo salar*, *Acanthopagrus schlegelii* and *Oncorhynchus mykiss* (Carmona-Antoñanzas *et al*., 2011; Jin *et al*., 2017b; Monroig *et al*., 2012; Oboh *et al*., 2017b; Zhao *et al*., 2019), and although in all cases Elovl4 participate in the biosynthesis of VLC-PUFA, the two isoforms do not have the same efficiency in converting the different substrates in all the species studied (Jin *et al*., 2017b; Monroig *et al*., 2010; Oboh *et al*., 2017b). As previously described in zebrafish (Monroig *et al*., 2010), *elovl4a* and *elovl4b* can present distinct substrate specificities, since Elovl4a has virtually no activity towards DHA itself, unlike Elovl4b. However, DHA in *Acanthopagrus schlegelii* was only

elongated by Elovl4a isoform (Jin *et al*., 2017b). Moreover, Elovl4 isoforms have different tissue distribution patterns, with *elovl4a* being mostly expressed in brain tissues (brain and pituitary) (Monroig *et al*., 2010; Oboh *et al*., 2017b), while *elovl4b* is located mostly in retina and gonads (Monroig *et al*., 2010; Oboh *et al*., 2017b). These spatio-temporal differences in the pattern of expression of both isoforms of *elovl4*, could be pivotal in early stages of development, where important changes at the physiological level are carried out in short periods of time (Zambonino-Infante and Cahu, 2001).

The rapid increase in expression values shown for *elovl4* (*elovl4a*, *elovl4b*) after hatching, besides the high *elovl5* transcript levels observed for *S. senegalensis* eggs, suggests that an over-expression of *elovl* genes is important to meet the high requirements of endogenous LC- and VLC-PUFA necessary for the optimal growth and development of neural tissue during early embryonic development independently of dietary supply (Morais *et al*., 2004). This pattern could be modified depending on the hypothetical requirements of VLC-PUFA associated to the larval development of each species, the conditions of the larval culture, as well as the physiological state of the fish. It is even possible that some maternal transference of target genes to the egg takes place to start the LC-PUFA biosynthesis in the embryo, thus the availability of PUFA for early neurogenesis could be ensured (Monroig *et al*., 2009; Morais *et al*., 2012).

Biometric parameters obtained for growth (TL, WW) of early and late larvae of both species showed higher growth performance for fish diets (live preys) containing high LC-PUFA (i.e., enriched). This may be due to a higher intake of the enriched live prey (rotifer and *Artemia*), since an intake of prey rich in LC-PUFA could activate the FA-detection system (hypothalamic mechanisms of lipid sensing that detect changes in plasma levels of LC-FA), positively regulating a higher food intake (Bonacic *et al*., 2016; Ibarra-Zatarain *et al*., 2015). Appetite and food intake are factors that greatly impact larval growth and development (Rønnestad *et al*., 2013), as they determine the amount of nutrients available to larvae for the high structural and energy demands for rapid growth and organogenesis (Bonacic *et al*., 2016; Hamre *et al*., 2013). It is known that lipids are an important source of metabolic energy, components of biological membranes and precursors of essential metabolites (Sargent *et al*., 1999). These properties are of particular importance in larvae of teleostean fish, which are characterized by extremely high growth rates coupled with high demands for energy and structural components (Conceição, 1997; Hamre *et al*., 2013; Tocher *et al*., 2010). The fatty acids released from lipid hydrolysis are used as energy substrates by the growing larvae, especially DHA (Hamre *et al*., 2013). Enriched diets used in our study were different both from a quantitative (higher lipid content) and qualitative (fatty acids) point of view than non-enriched diets, especially in ARA, EPA (*Artemia* E diet) n-6 DPA and DHA

levels (rotifer and *Artemia* E diet). These differences could be associated with the dissimilar growth performance shown in *S. aurata* and *S. senegalensis* early and late larvae fed the two different diets in our study, since there are numerous evidences that relate high contents of essential fatty acids, especially EPA and DHA, with optimal growth, survival, behavior and biological functions and processes in marine fish larvae (Hamre *et al*., 2013).

Nutritional regulation of *fads2* and *elovl5* have been extensively studied in fish (Izquierdo *et al*., 2008; Kuah *et al*., 2015; Li *et al*., 2016; Li *et al*., 2017; Morais *et al*., 2012). However, except for the studies in the crab *Scylla paramamosain* (Lin *et al*., 2018), in the fish *Larimichthys crocea* (Li *et al*., 2017) and in *Oncorhynchus mykiss* (Zhao *et al*., 2019), there are no studies on the nutritional regulation of *elovl4* in marine vertebrates. Delta-6 and Δ5-desaturase activity (capacity to bioconvert C_{18} precursors into PUFA) in fish responds to levels of PUFA present in the diet, over-expressing these enzymes to compensate a deficient supply of dietary PUFA (Izquierdo *et al*., 2008; Ren *et al*., 2012; Seiliez *et al*., 2003). However, in contrast with the results reported by Izquierdo *et al*. (2008), where a significant effect of dietary lipids on the regulation of Δ6 desaturase expression in gilthead seabream larvae was observed, no nutritional effects on *S. aurata fads2* and *elovl5* was detected in our study. Our results are in agreement with results reported by Geay *et al*. (2010), where the comparison between the two dietary groups revealed that the

use of a diet totally deprived of PUFA did not up-regulate the European sea bass *fads2* activity. Besides, these results are similar to those obtained in other marine fish species: e.g. Atlantic cod fed a PUFA free diet did not exhibit an increase of total desaturation/elongation activities (Tocher *et al*., 2006). This could be indicative of an insufficient Δ6 desaturase activity in the PUFA biosynthesis pathway to maintain the minimum requirements of EPA and DHA in *S. aurata* larvae (early and late larvae), which should be covered with a dietary supply of LC-PUFA. In agreement with results reported by Morais *et al*. (2012), our study showed that the *S. senegalensis fads2* but not *elovl5*, was up-regulated in response to low dietary LC-PUFA (non-enriched diet) in 40 dah larvae. These results are similar to those obtained in some freshwater fish species, e.g. silver barb, common carp and striped snakehead fed low PUFA diets, which exhibited an increase in total desaturation activity (Kuah *et al*., 2015; Nayak *et al*., 2017; Ren *et al*., 2012). This is probably due to the different desaturase activities shown by the Fads2 enzymes of each species, either Δ4 desaturase activity in *S. senegalensis*, or Δ6 activity in *S. aurata*, being the Δ4 desaturase activity the simplest and most direct pathway for the biosynthesis of DHA from EPA (Li *et al*., 2010; Morais *et al*., 2012). The up-regulation of Δ4 desaturase activity in visceral and head regions of *S. senegalensis* larvae as a consequence of a diet low in LC-PUFA could ensure that DHA levels remain constant under limited dietary DHA intake (Kuah *et al*., 2015; Morais *et al*., 2012). This could be indicative

of the importance of DHA production from EPA via the Δ4 desaturation step in order to maintain an optimal reserve of DHA in key (neuronal) tissues of carnivore fish (Kuah *et al*., 2015), suggesting the biological importance of this pathway to reduce LC-PUFA dietary dependence in *S. senegalensis*, compared to other marine fish like *S. aurata* (Morais *et al*., 2012).

Analyzing the results concerning the nutritional regulation of *elovl4*a and *elovl4b* genes in 16 dah larvae in response to dietary LC-PUFA, differences were observed in the expression of *elovl4a*, although only at the verge of statistical significance for *S. senegalensis*. *Elovl4a* was up-regulated in 16 dah larvae fed the enriched diet, whereas no differences were observed in the expression of *elovl4b* in response to different dietary regimes. Conversely, in 40 dah larvae, the expression pattern differed from the previous stage, showing a trend towards an over-expression for *elovl4b*, but not for *elovl4a*, in fish fed the enriched diet. This opposite effect of the two isoforms at different development stages (16 dah and 40 dah) of both species could be indicative of the different substrate specificity and tissue localization of *elovl4* isoforms (Monroig *et al*., 2010; Zhao *et al*., 2019). *Elovl4* seems to experience an up-regulation in the expression of one isoform or another, attending to the different demands of PUFA (LC- and VLC-PUFA) faced in function of the stage and the degree of fish tissue development. In contrast with the results observed in other fishes (Li *et al*., 2017; Zhao *et al*., 2019), this over-expression responds to a scenario

(high levels of substrate) where there is enough dietary availability of LC-PUFA (essentially n-6 DPA, EPA and DHA), which could suggest that both isoforms respond positively to high levels of LC-PUFA activating its transcription to support the formation of specific tissues that have high requirements for VLC-PUFA (Monroig *et al*., 2011). In accordance with Li *et al*. (2017) and Zhao *et al*. (2019), the highest levels of *elovl4a* and *elovl4b* expression, shown in the head (probably in eyes and brain) where VLC-PUFA have a key biological function (Xue *et al*., 2014), are probably linked to this need. This up-regulation can be especially important in predatory fish that need excellent cognitive traits, especially those with a strong nocturnal activity, like *S. senegalensis* (Navarro *et al*., 2009). The lower expression of *elovl4b* in response to low levels of dietary LC-PUFA in late larvae may be associated to local (organ, tissue) synthesis of VLC-PUFA only if adequate levels of precursors (LC-PUFA) are reached, and deserves further exploration.

Although VLC-PUFA were not measured due to the analytical difficulty and the predicted low concentrations existing in the tissues of the species under study (Garlito *et al*., 2019) we can conclude that the presence of *elovl4a* and *elovl4b* mRNA transcripts in embryos and larval fish, including the eggs before hatching, suggests that VLC-PUFA biosynthesis can be important in early development. These findings highlight the importance that the study of VLC-PUFA and their biosynthesis might have in farmed fish in which altered visual acuity (critical in

visual predators such as most cultured fish species, especially during larval stages) and disruptions of brain functioning can jeopardize their normal development (Monroig *et al*., 2010). Both isoforms of *elovl4* are expressed preferentially in the head, likely associated to the hypothetical abundance of VLC-PUFA in fish neural tissues including retina. Moreover, the results for both species suggest that the expression of *elovl4* (isoform *a* in early larvae, and *b* in late larvae) can be regulated positively according to the dietary content of LC-PUFA in early stages, including the potential activation of the VLC-PUFA biosynthesis during short-term feeding periods (seven days). These results can be very helpful in the design of diets for larvae (early and late stages) of *S. aurata* and *S. senegalensis*, opening the possibility to make feasible an early nutritional programming along the larval rearing including short periods, particularly for *S. senegalensis*, since the low LC-PUFA requirements attributed to this species could be reconsidered as a tool for the activation of *elovl4* genes, which can be necessary for the maintenance of optimal levels of VLC-PUFA at these stages.

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CAPÍTULO 5:

Nutritional regulation of genes responsible for long-chain (C_{20-24}) and very long-chain ($>C_{24}$) polyunsaturated fatty acid biosynthesis in post-larvae of gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*)

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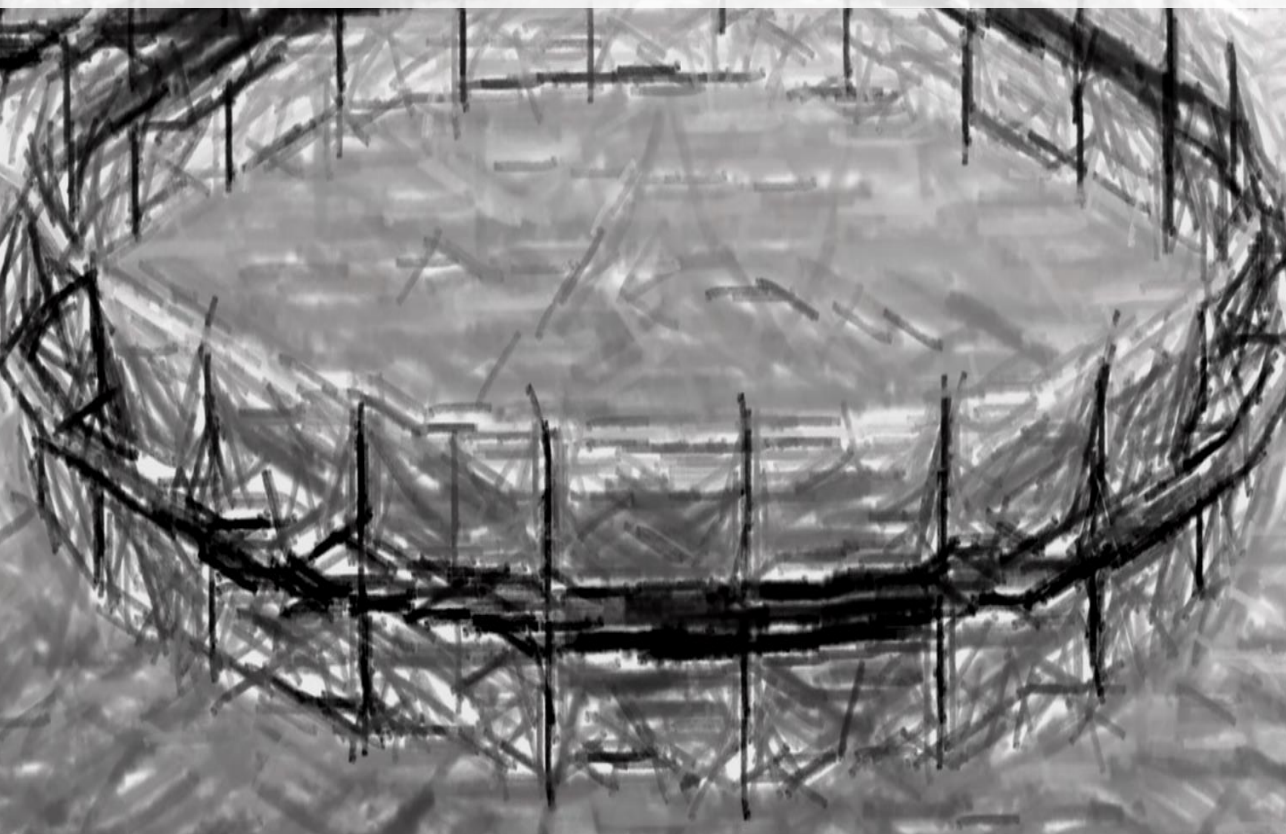

Abstract

The fatty acyl elongases Elovl4 are pivotal components in the biosynthesis of very long-chain polyunsaturated fatty acids (VLC-PUFA) from long-chain polyunsaturated fatty acids (LC-PUFA). Thus, nutritional regulation of Elovl4, as well as other elongase and desaturase genes involved in LC-PUFA biosynthesis (e.g., Elovl5, Fads2) has been proposed as a strategy to enhance endogenous production of LC-PUFA and VLC-PUFA under intensive farming conditions. This study aimed at investigating the nutritional regulation of genes involved in the biosynthesis of VLC-PUFA (*elovl4* isoforms *a* and *b*) and LC-PUFA (*fads2*, *elovl5*) in *Sparus aurata* and *Solea senegalensis* post-larvae fed three inert micro-diets with graded concentrations of dietary LC-PUFA by using different combinations of fish oil and soya oil. The effect of dietary LC-PUFA on survival, growth and fatty acid composition was also examined. The results denoted that, while no effects were observed in survival, fish fed the diet with the highest LC-PUFA content during 30 d showed a higher total length and wet weight. Gene expression results showed that *fads2*, *elovl5*, *elovl4a* and *elovl4b* can be regulated by dietary LC-PUFA content. Remarkably, our results denoted a differential *elovl4* nutritional regulation associated to each species. The head is the body part studied with the highest *elovl4* transcripts in both fish species.

Keywords: *Elovl4*; Marine post-larvae; Nutritional regulation; *Solea senegalensis*; *Sparus aurata*; Very long-chain polyunsaturated fatty acid.

5.1. Introduction

Marine teleost farming has been traditionally based on diets containing high levels of long-chain polyunsaturated fatty acids (LC-PUFA; C_{20-24}), associated with the inclusion of fish oil (FO) and, to a lesser extent, fishmeal, known as "marine ingredients" that are mostly derived from capture fisheries (Benedito-Palos *et al*., 2008; Tocher, 2015). While this strategy guaranteed growth and health of farmed fish, the continuous expansion of aquaculture and the finite nature of marine ingredients have urged aquaculture to seek for alternative ingredients (Shepherd *et al*., 2017; Watanabe, 2002). Vegetable oils (VO) are now extensively used in aquafeed formulations (Jobling, 2016; Turchini *et al*., 2011). However, the replacement of FO with VO in aquafeeds entails a reduction of the dietary supply of LC-PUFA to marine fish (Houston *et al*., 2017; Tocher, 2015), which negatively impacts the product quality due to the reduced health promoting n-3 LC-PUFA content (Tocher, 2015), as well as undesirable effects on the metabolism, composition and growth of marine fish (Houston *et al*., 2017). Early life-cycle stages are particularly sensitive to low availability of pre-formed dietary LC-PUFA due to the rapid development of neural tissues that accumulate large amounts of these compounds (Hamre *et al*., 2013; Izquierdo *et al*., 2015).

Some LC-PUFA, such as arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), are considered as

essential nutrients for marine fish, thus they must be supplied through the diet to prevent deficiency symptoms (Monroig *et al*., 2018; Tocher, 2015). They are involved in relevant biological processes, keeping a key role as essential components on neural (Dyall, 2015) and retinal membranes (Gawrisch *et al*., 2003) of higher vertebrates, besides being precursors of several autocrine signaling molecules (Houston *et al*., 2017; Serhan *et al*., 2008) and longer fatty acids, as very long-chain (>C_{24}) polyunsaturated fatty acid (VLC-PUFA) (Deák *et al*., 2019). VO are rich in C_{18} polyunsaturated fatty acids (PUFA) such as α-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6). C_{18} PUFA, while not playing vital roles in vertebrates *per se*, are the precursors of the physiologically active LC-PUFA, ARA, EPA and DHA (Monroig *et al*., 2018). The biosynthesis of LC-PUFA from C_{18} PUFA varies among vertebrate species (Castro *et al*., 2016; Monroig *et al*., 2018) and is dependent upon their gene repertoire and function of the encoded elongation of very long-chain fatty acid (Elovl) and fatty acyl desaturase (Fads) enzymes (Castro *et al*., 2016; Monroig *et al*., 2018).

With the exception of basal species like eels (Lopes-Marques *et al*., 2018), virtually all teleost fish desaturases are considered *fads2* orthologs (Castro *et al*., 2016). However, teleost Fads2 have a remarkable functional diversity, having Δ4, Δ5, Δ6 and Δ8 desaturase activities and thus enabling alternative routes within the LC-PUFA biosynthesis (Castro *et al*., 2016; Monroig *et al*., 2018). Among Elovl

enzymes, Elovl2, Elovl4 and Elovl5 participate in PUFA elongation and they are representatives from the three types of fatty acyl elongases that have been investigated in teleosts (Castro *et al*., 2016; Monroig *et al*., 2018). Elovl2 and Elovl5 have a common evolutionary origin (Monroig *et al*., 2016) and, consequently, share to some extend substrate preference since both enzymes can elongate C_{18-22} PUFA substrates (Monroig *et al*., 2018). Nevertheless, the *elovl2* gene appears to be absent in most marine farmed fish (Castro *et al*., 2016; Monroig *et al*., 2018).

Teleosts possess two isoforms of Elovl4 termed Elovl4a and Elovl4b (Monroig *et al*., 2010). These enzymes are pivotal in the biosynthesis of VLC-PUFA through elongation of LC-PUFA substrates in mammals (Deák *et al*., 2019) or in fish (Monroig *et al*., 2010). VLC-PUFA play important roles in vision, brain function and reproduction of mammals (Agbaga *et al*., 2010; Aldahmesh *et al*., 2011; Deák *et al*., 2019; Furland *et al*., 2007; Mandal *et al*., 2004; Poulos, 1995). However, VLC-PUFA have been barely investigated in fish due, among other reasons, to their low abundance and analytical complexity (Agbaga *et al*., 2010; Garlito *et al*., 2019), and their research has been mostly restricted to the molecular and functional characterization of Elovl4 enzymes responsible for VLC-PUFA biosynthesis (Betancor *et al*., 2020; Carmona-Antoñanzas *et al*., 2011; Jin *et al*., 2017b; Kabeya *et al*., 2015; Li *et al*., 2017a, 2017b; Monroig *et al*., 2010; Oboh *et al*., 2017b; Zhao *et al*., 2019). Further research on VLC-PUFA biosynthesis in fish has focused on the

study of molecular mechanisms involved in nutritional regulation of *elovl4* in order to understand the impacts that current aquafeed formulations with reduced levels of LC-PUFA, biosynthetic precursors of VLC-PUFA, can have on farmed species (Betancor *et al*., 2020; Li *et al*., 2017a, 2017b; Monroig *et al*., 2011a, Zhao *et al*., 2019). Such impacts can be also dependent upon the capacity that each species has to endogenously produce the precursors of VLC-PUFA (LC-PUFA) themselves and, consequently, it is interesting to investigate regulatory mechanisms from a comparative perspective.

The gilthead seabream (*Sparus aurata*) and the Senegalese sole (*Solea senegalensis*) are both marine teleosts with substantial differences in their capacity to biosynthesize LC-PUFA. While both species possess Elovl5 elongases (Agaba *et al*., 2005; Morais *et al*., 2012), they differ in the function of their fatty acyl desaturase Fads2. On one hand, *S. aurata* has a Fads2 with primarily Δ6 desaturase activity (Seiliez *et al*., 2003; Zheng *et al*., 2004). On the other, *S. senegalensis* has a Fads2 with Δ4 activity (Morais *et al*., 2012). Such differences determine their ability to biosynthesize DHA, with a more direct route "Δ4 pathway" operated in *S. senegalensis* and the more complex "Sprecher pathway" of *S. aurata* (Oboh *et al*., 2017a). Along these enzymatic differences in LC-PUFA biosynthesis, their specific larval development and different feeding requirements and habits (pelagic *vs* benthonic) also make *S. aurata* and *S. senegalensis* interesting models for nutritional

regulation of *fads* and *elovl* genes involved in the biosynthesis of physiologically important compounds such as LC-PUFA and VLC-PUFA. In a previous study, we have investigated the temporal expression patterns and the nutritional regulation, of the genes involved in the biosynthesis of VLC-PUFA (*elovl4a*, *elovl4b*) and their precursors, LC-PUFA (*fads2*, *elovl5*), during early larval stages of both species (Torres *et al*., 2020). Therefore, following the research line along of the farming cycle of the cited species, this study aimed at investigating the nutritional regulation of the above described genes in *S. aurata* and *S. senegalensis* post-larvae (weaned post-metamorphic larvae) fed three inert micro-diets with graded concentrations of dietary LC-PUFA. Moreover, the effects of dietary LC-PUFA on growth and fatty acid (FA) composition were also examined.

5.2. Materials and methods

5.2.1. Fish culture and dietary treatments

Post-larvae of *S. aurata* and *S. senegalensis* were supplied by *Piscicultura Marina Mediterránea* S.L. (Castellón, Spain) and Stolt Sea Farm S.A. (A Coruña, Spain), respectively, with an initial total length/wet weight of 1.80 cm/0.08 g for seabream, and 1.36 cm/0.03 g for sole. Fish were cultured at 18-20 °C in seawater (salinity of 37.5 ± 0.5 g l^{-1}) controlling NO_2 and NO_3 contents, with oxygen to saturation, continuous flow-through and a 12L:12D photoperiod. A total of 450 post-

larvae of each species, previously acclimated for 7 d, were randomly distributed in nine 20 l cylindrical tanks (50 individuals each) corresponding to three independent replicates of three dietary treatments with an initial fish biomass of stocking, calculated by (number of fish / l) x average wet weight (WW), of 0.2 g l^{-1} and 0.07 g l^{-1} for *S. aurata* and *S. senegalensis*, respectively. Post-larvae were fed the corresponding diet to apparent visual satiation (*ad libitum*) three times daily (09:00, 14:00 and 20:00 h) for 30 d. The experimental diets, designed and manufactured by Lifebioencapsulation S.L. (Almeria, Spain) using standard aquafeed procedures, i.e. mixing ingredients, inclusion of feed additives, gentle extrusion with temperature control, and granulation within the size range of experimental feed, consisted of three inert micro-diets (0.5-0.8 mm). The three diets were formulated with different levels combining FO and soya oil (SO), resulting in different FA profiles, and consequently high (Diet 1), medium (Diet 2) and low (Diet 3) LC-PUFA contents. The ingredients and proximate composition of the experimental diets are shown in Table 5.1. Table 5.2 includes the fatty acid composition of the experimental diets.

Table 5.1. Ingredients and proximate composition of the experimental diets used in the present study.

Ingredients (g/kg diet)	Diet 1	Diet 2	Diet 3
Fish meal (LT94)	151	151	151
Lysine	11	11	11
Methionine	5	5	5
Squid meal	50	50	50
Fish soluble protein concentrate [1]	50	50	50
Krill meal	30	30	30
Corn gluten meal	155	155	155
Soybean meal[2]	379	379	379
Hydrolyzed yeast[3]	5	5	5
Concentrated nucleotides[4]	1	1	1
Fish oil	102	33	5
Soya oil	0	69	97
Soya lecithin	10	10	10
Maltodextrin	5	5	5
Choline chloride	5	5	5
Betaine	5	5	5
Vitamin and minerals premix	19	19	19
L-Ascorbyl-2-monophosphate-Na[5]	1	1	1
Guar gum	10	10	10
Alginate	10	10	10
Proximate composition			
Dry matter (DM, %)	94.36	94.34	94.60
Crude protein (% DM)	56.17	55.14	56.32
Crude Fat (% DM)	18.37	18.13	18.12
Ash (% DM)	6.22	6.35	6.33
EPA (mg/g DM)	10.96	7.57	4.07

DHA(mg/g DM)	12.59	9.42	5.38
EPA+DHA(mg/g DM)	23.55	16.99	9.45
Gross energy (MJ/kg DM)[a]	18.83	18.83	18.83

[a] Calculated according to gross energy values for protein (16.7 kJ/g), fat (37.4 kJ/g), and carbohydrate (16.7 kJ/g) based on general Atwater factors according to: (((16.74 x g of protein) + (37.4 x g of lipids) + (16.7 x g of carbohydrates)) x 10)/ 1000**.**

(% DM): percentage of components with respect to the total dry matter; EPA (20:5n-3): eicosapentaenoic acid; DHA (22:6n-3): docosahexaenoic acid.

[1]CPSP90 (British Aqua Feeds Ltd., Goole, UK)

[2]Soycomil (Sime Darby Unimills B.V., Zwijndrecht, The Netherlands)

[3]Celmanax (Arm & Hammer Animal Nutrition, New Jersey, USA)

[4]Nucleoforce Fish (Bioiberica S.A.U., Barcelona, Spain)

[5]Stay C Roche 0.2 % (Roche Farma S.A., Madrid, Spain)

Table 5.2. Fatty acid composition of the experimental diets used (% of total fatty acids). Results are expressed as mean ± SD (n = 3).

Fatty acid	Diet 1	Diet 2	Diet 3
14:0	4.62 ± 0.24	2.37 ± 0.05	1.36 ± 0.02
16:0	16.93 ± 0.12	14.14 ± 0.06	12.92 ± 0.02
16:1n-7	5.06 ± 0.06	2.47 ± 0.02	1.33 ± 0.02
16:1n-9	0.21 ± 0.01	0.13 ± 0.02	0.08 ± 0.03
16:2	0.30 ± 0.00	0.14 ± 0.00	0.07 ± 0.00
16:3n-3	0.48 ± 0.01	0.19 ± 0.01	0.09 ± 0.01
16:4n-3	0.88 ± 0.02	0.40 ± 0.01	0.19 ± 0.00
18:0	3.16 ± 0.02	3.86 ± 0.01	4.15 ± 0.02
18:1n-5	0.18 ± 0.00	0.12 ± 0.00	0.09 ± 0.00
18:1n-7	2.56 ± 0.01	2.06 ± 0.01	1.82 ± 0.03

18:1n-9	12.92 ± 0.06	18.39 ± 0.07	20.60 ± 0.06
18:2n-6	8.52 ± 0.13	29.73 ± 0.05	38.81 ± 0.05
18:3 n-6	0.18 ± 0.01	0.08 ± 0.01	0.04 ± 0.00
18:3n-3	1.45 ± 0.01	3.57 ± 0.02	4.52 ± 0.05
18:4n-3	2.13 ± 0.01	1.01 ± 0.01	0.54 ± 0.00
20:0	0.26 ± 0.00	0.34 ± 0.01	0.37 ± 0.00
20:1n-7	0.20 ± 0.00	0.11 ± 0.00	0.07 ± 0.00
20:1n-9	3.86 ± 0.02	2.11 ± 0.01	1.37 ± 0.00
20:1n-11	0.51 ± 0.01	0.32 ± 0.01	0.21 ± 0.00
20:2n-6	0.24 ± 0.00	0.15 ± 0.00	0.11 ± 0.00
20:3n-6	0.09 ± 0.00	0.04 ± 0.00	-
20:4n-6	0.78 ± 0.00	0.37 ± 0.00	0.18 ± 0.03
20:3n-3	0.14 ± 0.00	0.08 ± 0.01	0.06 ± 0.00
20:4n-3	0.57 ± 0.00	0.27 ± 0.00	0.14 ± 0.00
20:5n-3	9.86 ± 0.08	4.81 ± 0.03	2.72 ± 0.01
22:0	0.15 ± 0.00	0.28 ± 0.00	0.34 ± 0.00
22:1n-9	0.55 ± 0.01	0.29 ± 0.03	0.20 ± 0.00
22:1n-11	5.31 ± 0.03	2.56 ± 0.04	1.41 ± 0.01
22:4n-6	0.09 ± 0.00	0.04 ± 0.00	-
22:5n-6	0.31 ± 0.01	0.13 ± 0.00	0.06 ± 0.00
22:5n-3	1.10 ± 0.01	0.50 ± 0.00	0.25 ± 0.00
22:6n-3	11.25 ± 0.10	5.96 ± 0.08	3.58 ± 0.27
24:0	0.09 ± 0.00	0.12 ± 0.00	0.13 ± 0.00
n-3 PUFA	27.86 ± 0.24	16.79 ± 0.17	12.09 ± 0.34
n-6 PUFA	10.21 ± 0.15	30.54 ± 0.06	39.20 ± 0.08
n-3 LC-PUFA	22.92 ± 0.19	11.62 ± 0.13	6.75 ± 0.28
n-6 LC-PUFA	1.51 ± 0.01	0.73 ± 0.00	0.35 ± 0.03
FAME (mg/g)	111.07 ± 4.50	157.80 ± 12.16	150.87 ± 5.22

PUFA: Polyunsaturated fatty acids; LC-PUFA: Long-chain polyunsaturated fatty acids; (-): not detected; FAME: Fatty acid methyl ester.

5.2.2. Fish growth and survival

Collection of biological samples and measurements of individual total lengths (TL) and WW of post-larvae were carried out at 15 and 30 d after the start of the feeding trial. Post-larvae TL was measured manually with an ichthyometer, and WW was determined using a Mettler Toledo XS105 semi-microbalance (Mettler-Toledo S.A.E., Barcelona, Spain). Measurements of TL and WW were carried out in four individuals per replicate (n = 12 per dietary treatment). Survival was estimated by counting the total number of live post-larvae/total fish stocked x 100 (%). Dead fish, differentiated by dietary treatments, were recorded daily and removed.

All fish were fasted for 24 hours previously to sampling and then immersed in ice water (anesthetic method) and immediately sacrificed by cervical dislocation. Fish samples, after measured and weighed, were immediately frozen and kept at -80 °C until further dissection and analysis. Previous to FA and gene expression analysis, head, visceral mass (digestive system, liver, spleen, and swim bladder) and muscle were dissected and processed separately. For FA analysis, only the head samples were processed due to the accumulation of LC-PUFA and VLC-PUFA in neural tissues pointed out above (Dyall, 2015; Gawrisch *et al*., 2003).

5.2.3. Fatty acid analysis

Total lipids of the experimental diets (n = 3) and post-larvae heads (n = 4) from each dietary treatment were extracted with chloroform/methanol (2:1, v/v) according to Folch *et al*. (1957) and quantified gravimetrically after evaporation of the solvent under nitrogen flow, followed by vacuum desiccation overnight. Total lipids were resuspended at 10 mg ml^{-1} in chloroform/methanol (2:1) containing 0.01 % (w/v) butylhydroxytoluene (BHT). Then, 50 µl of total lipids were subjected to an acid-catalyzed transesterification (Christie, 1982). Fatty acid methyl esters (FAME) were subsequently purified as previously described by Monroig *et al*. (2013).

FA composition was determined using a Thermo Scientific TRACE GC Ultra gas chromatograph (Thermo Fisher Scientific, Madrid, Spain), equipped with a fused silica 30 m × 0.25 mm open tubular column (Tracer, TRB-WAX, film thickness: 0.25 µm, Teknokroma, Barcelona, Spain). Injections of 1 µl samples were carried out on-column, using helium as carrier gas (1.5 ml min^{-1} constant flow), and a thermal gradient from 50 (injection temperature) to 220 °C, and reported as % of total fatty acids. FAME were identified by comparison with known standards.

5.2.4. RNA extraction and real time quantitative PCR (qPCR)

Total RNA was isolated from head, viscera and muscle of post-larvae fed the experimental diets (n = 12) using Maxwell 16 LEV simplyRNA Tissue Kit (Promega

Biotech Ibérica S.L., Madrid, Spain) following the manufacturer's instructions. RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop ND-2000C, Thermo Fisher Scientific). Two µg of total RNA per sample were reverse transcribed into cDNA using the M-MLV reverse transcriptase first strand cDNA synthesis kit (Promega Biotech Ibérica S.L.) following manufacturer's instructions, using a mixture (3:1, v/v) of random primers and anchored oligo $(dT)_{15}$ primer (Promega Biotech Ibérica S.L.). Expression of the fatty acyl desaturase *fads2* and the elongases *elovl5*, *elovl4a* and *elovl4b* was quantified by qPCR using the primers shown in Table 5.3. Primers were designed using Primer3 software (http://primer3.sourceforge.net) (Rozen and Skaletsky, 2000). The amplification efficiency of the primer pairs was assessed by serial dilutions of standard solutions of the studied genes with known copy numbers that helped to build a standard curve, which also allowed the conversion of threshold cycle (Ct) values to copy numbers. Amplifications were carried out in technical duplicates on a qPCR thermocycler (CFX Connect Real-Time System, Bio-Rad Laboratories S.A., Madrid, Spain) in reactions with a final volume of 20 µl, containing 5 µl diluted (1/20) cDNA as template for all genes, except for the *S. senegalensis β-actin* (*actb*) gene (1/200), 0.5 µl of each primer (0.25 µM) and 4 µl Master Mix qPCR No-ROX PyroTaq EvaGreen 5x (CMB-Bioline, Madrid, Spain). All runs included a systematic negative control consisting of a non-template control (NTC). The qPCR program consisted of

an initial activation step at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, elongation at 72 °C for 15 s, and a final melt curve of 0.5 °C increments from 60 °C to 90 °C, enabling confirmation of the amplification of a single product in each reaction. Three potential reference genes (*β-actin*, *elongation factor 1α* and *18s rRNA*) were tested and, after checking their stability using the Genorm software (Vandesompele *et al*., 2002), *β-actin* was chosen for normalization of the candidate gene expression. Gene expression results are given as mean normalized values ± standard deviation (SD) corresponding to the ratio between copy numbers of fatty acyl desaturase (*fads2*) and fatty acyl elongase (*elovl5*, *elovl4a* and *elovl4b*) transcripts and copy numbers of the reference gene *β-actin* (*actb*).

Table 5.3. Primers used for real-time quantitative PCR (qPCR) of *S. aurata* and *S. senegalensis* genes. Sequences of the primer pairs used (Forward: F; Reverse: R), annealing temperatures (Ta) of the primer pairs, size of the fragments produced, and accession number of the sequences used for the primer design are shown.

Sparus aurata					
Transcript	**Primer**	**Primer sequence**	**Ta**	**Fragment**	**Accession No**
elovl4a	F	5'-GCCCAAGTACATGAAGAACAGAG-3'	60 °C	169 bp	MK610320
	R	3'-GGGGTCGTCTGAGTAGTCCA-5'			
elovl4b	F	5'-GTCAAGTACTCCAACGATGTCAA-3'	60 °C	247 bp	MK610321
	R	3'-TGAGCACATGGATGGAAGAG-5'			
elovl5	F	5'-TCGTCCACGTCGTGATGTAT-3'	60 °C	152 bp	Q68YU3
	R	3'-ACATGGCCATATGACTGCAA-5'			
fads2	F	5'-CACTCAGCCAGTCGAGTACG-3'	60 °C	199 bp	GQ162822
	R	3'-ACAGCACAGGTAGCGAAGGT-5'			
Actb	F	5'-TGCGTGACATCAAGGAGAAG-3'	60 °C	190 bp	X89920
	R	3'-CAGGACTCCATACCGAGGAA-5'			

Solea senegalensis					
Transcript	**Primer**	**Primer sequence**	**Ta**	**Fragment**	**Accession No**
elovl4a	F	5'-AGGTGAGGTAGGGCCTTGTT-3'	60 °C	220 bp	MN164537
	R	3'-TGAAAACAGCCACCTTAGGC-5'			
elovl4b	F	5'-CCTCTGCCTTGTCCAGTTTC-3'	60 °C	175 bp	MN164625
	R	3'-CAATTTGATGCCCAGTTCCT-5'			
elovl5	F	5'-CAAGTACATGCAGCACAGGC-3'	60 °C	116 bp	JN793448
	R	3'-GCCACACAGCACTAACAAGC-5'			
fads2	F	5'-GTTCGTGTGGGTGACTCAGA-3'	60 °C	121 bp	JN673546
	R	3'-GTCGTTGAAGGAGGACTGCT-5'			
Actb	F	5'-ACAATGAGCTGAGAGTCGCC-3'	60 °C	132 bp	DQ485686
	R	3'-CAACATACATGGCGGGGGTA-5			

5.2.5. Statistical analysis

All data were checked for homogeneity of variances using Levene's test. TL, WW and FA composition data of post-larvae fed the different experimental diets during the same feeding period, i.e. 15 or 30 d, were analyzed by one-way analysis of variance (ANOVA, $P \leq 0.05$) followed by Tukey HSD *post-hoc* test. Gene expressions among body parts (with the three diets pooled) and among diets (with the three body parts pooled) were compared with a one-way analysis of variance (ANOVA, $P \leq 0.05$) followed by Tukey HSD *post-hoc* test. The results are presented as means ± SD. The statistical software SPSS 24.0 (SPSS Inc., Chicago, USA) was used to analyze the data.

5.3. Results

5.3.1. Survival

S. aurata final survivals for fish fed Diet 1, Diet 2 and Diet 3, were 96.5 %, 97.5 % and 98.3 %, respectively (**Figure 5.1 A**). *S. senegalensis* final survivals for fish fed Diet 1, Diet 2 and Diet 3, were 92.6 %, 93.9 % and 92.2 %, respectively (**Figure 5.1 B**). The final *S. aurata* biomass of stocking was 1.53 g l^{-1}, 1.16 g l^{-1} and 0.78 g l^{-1} for fish fed Diet1, Diet 2 and Diet 3, respectively. For *S. senegalensis*, the final post-larvae biomass of stocking was 0.25 g l^{-1}, 0.13 g l^{-1} and 0.08 g l^{-1} for fish fed Diet1, Diet 2 and Diet 3, respectively.

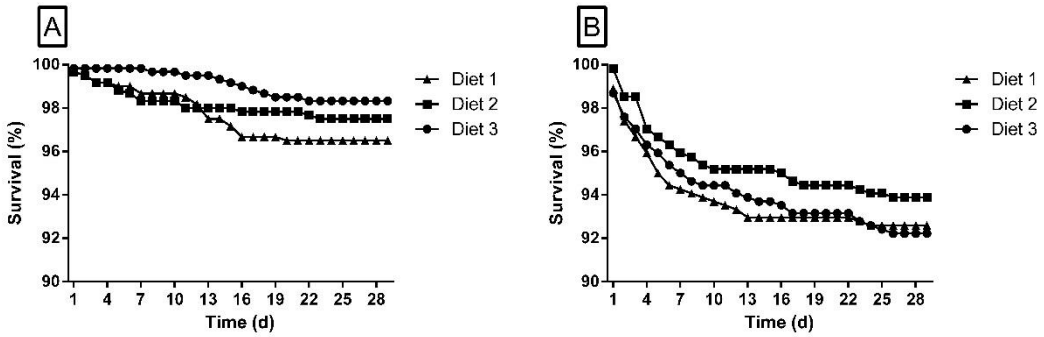

Figure 5.1. Accumulated daily survival (live fish/ total fish*100) of *S. aurata* (**A**) *and S. senegalensis* (**B**) post-larvae fed with different experimental diets (Diet 1, Diet 2 and Diet 3) during the study period.

5.3.2. Biometric parameters

For *S. aurata*, at 15 d of feeding, no significant differences were found in TL and WW among dietary treatments. However, after 30 d, *S. aurata* fed Diet 1 presented higher growth performance (TL and WW) than fish fed Diet 3 (**Figure 5.2**). In *S. senegalensis*, after 15 d of feeding, no significant differences were observed in biometric parameters (TL and WW) among fish from the different dietary treatments. At 30 d, *S. senegalensis* fed Diet 1 presented higher growth performance (TL and WW) than fish fed Diets 2 and 3 (**Figure 5.3**).

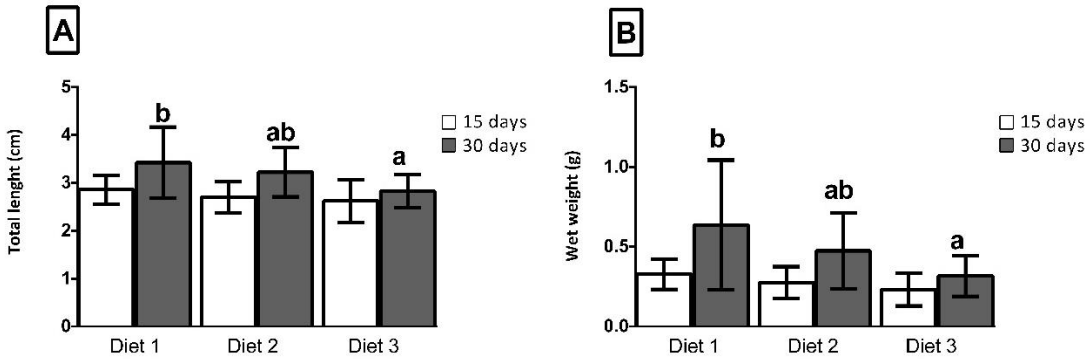

Figure 5.2. Total length (**A**) and wet weight (**B**) of *S. aurata* post-larvae fed with different experimental diets (Diet 1, Diet 2 and Diet 3). Values are presented as mean ± SD (n = 12). Different letters above the columns show significant differences (one way-ANOVA and Tukey test, $P \leq 0.05$) among fish fed with different diets for the same feeding period (15 and 30 d).

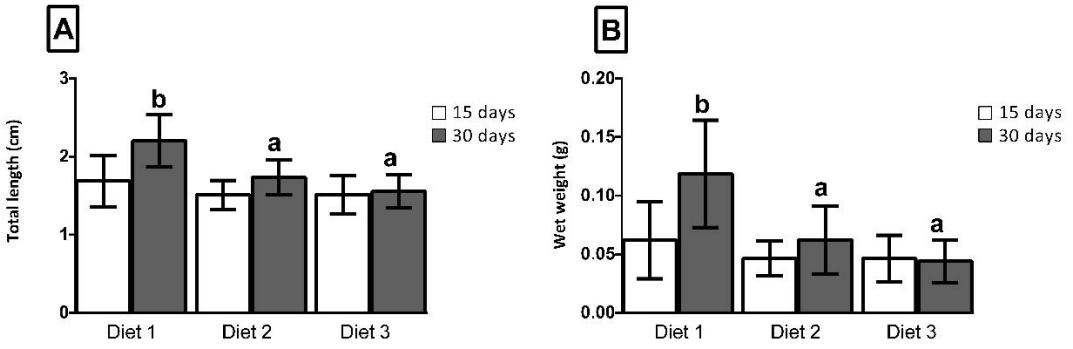

Figure 5.3. Total length (**A**) and wet weight (**B**) of *S. senegalensis* post-larvae fed with different experimental diets (Diet 1, Diet 2 and Diet 3). Values are presented as mean ± SD (n = 12). Different letters above the columns show significant differences (one way-ANOVA and Tukey test, $P \leq 0.05$) among fish fed with different diets for the same feeding period (15 and 30 d).

5.3.3. Fatty acid composition

5.3.3.1. S. aurata

No significant differences were found in the total lipid amount of heads from *S. aurata* fed Diets 1, 2 and 3. At 15 d, no significant differences in 22:6n-3 amount were observed among fish fed the three diets. However, *S. aurata* fed Diet 1 showed higher levels of 18:4n-3, 20:4n-3, 20:5n-3 and 22:5n-3 compared to fish fed Diets 2 and 3. Fish fed Diet 1 showed lower amounts of total n-6 PUFA (18:2n-6, 18:3n-6, 20-2n-6 and 20:3n-6) than fish fed Diets 2 and 3 (Table 5.4). At 30 d of feeding, *S. aurata* fed Diet 1 showed higher levels of total n-3 PUFA (18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3) than fish fed Diets 2 and 3 (Table 5.4). For total n-6 PUFA

an amount gradient associated with diet is observed, since *S. aurata* fed Diets 1and 3 showed the lowest and highest levels of n-6 PUFA, respectively (Table 5.4). *S. aurata* fed Diet 1 showed a lower amount of 18:2n-6, 18:3n-6, 20:2n-6 and 20:3n-6 FA than fish fed Diets 2 and 3.

Table 5.4. Selected fatty acid content (% of total fatty acids) of the total lipids from the head of *S. aurata* post-larvae fed experimental diets, with different PUFA content, during 15 and 30 d. Results are expressed as mean ± SD (n = 4). Different superscripts denote significant differences among diets in each sampling point (one way-ANOVA and Tukey test, $P \leq 0.05$).

	S. aurata					
	15 d			**30 d**		
Fatty acid	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 1**	**Diet 2**	**Diet 3**
18:2n-6	7.54 ± 0.53^{a}	18.01 ± 3.34^{b}	19.81 ± 4.49^{b}	7.18 ± 1.08^{a}	20.25 ± 0.35^{b}	25.86 ± 2.34^{c}
18:3n-6	0.14 ± 0.01^{a}	1.55 ± 0.24^{b}	1.65 ± 0.86^{b}	0.13 ± 0.02^{a}	1.86 ± 0.31^{b}	2.65 ± 0.74^{b}
20:2n-6	0.20 ± 0.01^{a}	0.27 ± 0.03^{b}	0.34 ± 0.04^{c}	0.18 ± 0.01^{a}	0.23 ± 0.01^{b}	0.29 ± 0.04^{c}
20:3n-6	0.16 ± 0.05^{a}	0.25 ± 0.03^{ab}	0.37 ± 0.14^{b}	0.13 ± 0.02^{a}	0.22 ± 0.06^{a}	0.36 ± 0.07^{b}
20:4n-6	1.19 ± 0.06	0.94 ± 0.30	1.08 ± 0.35	1.18 ± 0.21^{b}	0.69 ± 0.11^{a}	0.55 ± 0.10^{a}
22:5n-6	0.40 ± 0.06	0.28 ± 0.12	0.37 ± 0.03	0.34 ± 0.06^{b}	0.18 ± 0.04^{a}	0.13 ± 0.05^{a}
18:3n-3	0.81 ± 0.10	1.35 ± 0.39	1.23 ± 0.46	0.77 ± 0.17^{a}	1.64 ± 0.07^{b}	2.07 ± 0.43^{b}
18:4n-3	0.85 ± 0.07^{c}	0.42 ± 0.13^{b}	0.17 ± 0.03^{a}	0.84 ± 0.22^{b}	0.49 ± 0.09^{a}	0.43 ± 0.13^{a}
20:4n-3	0.34 ± 0.03^{b}	0.19 ± 0.06^{a}	0.11 ± 0.01^{a}	0.31 ± 0.05^{b}	0.14 ± 0.02^{a}	0.10 ± 0.00^{a}
20:5n-3	6.01 ± 0.64^{c}	3.30 ± 0.27^{b}	1.86 ± 0.15^{a}	5.29 ± 0.36^{b}	2.77 ± 0.71^{a}	2.11 ± 0.36^{a}
22:5n-3	1.22 ± 0.06^{c}	0.73 ± 0.06^{b}	0.51 ± 0.06^{a}	1.10 ± 0.08^{b}	0.56 ± 0.15^{a}	0.45 ± 0.05^{a}
22:6n-3	19.76 ± 1.17	17.88 ± 4.56	15.90 ± 2.07	19.68 ± 2.65^{b}	12.86 ± 3.64^{a}	13.60 ± 3.45^{ab}
Total n-3 PUFA	28.99 ± 2.07^{b}	23.87 ± 5.44^{ab}	19.78 ± 2.78^{a}	27.98 ± 3.54^{b}	18.47 ± 4.69^{a}	18.76 ± 4.42^{a}
Total n-6 PUFA	9.64 ± 0.72^{a}	21.32 ± 4.05^{b}	23.63 ± 6.11^{b}	9.14 ± 1.40^{a}	23.41 ± 0.88^{b}	29.84 ± 3.34^{c}
Total unsaturates	65.45 ± 6.02	70.70 ± 16.16	66.21 ± 11.57	65.29 ± 8.51^{a}	68.92 ± 8.68^{b}	72.87 ± 10.53^{b}
Total saturates	29.29 ± 1.84	29.74 ± 2.76	33.56 ± 5.81	31.64 ± 2.94^{b}	29.54 ± 2.96^{ab}	26.26 ± 2.05^{a}
Total MUFA	26.25 ± 3.03^{b}	23.74 ± 3.81^{ab}	21.42 ± 1.48^{a}	27.27 ± 3.23	26.50 ± 3.05	23.76 ± 2.65
Total PUFA	39.20 ± 2.99	46.96 ± 10.36	44.78 ± 10.09	38.08 ± 5.28^{a}	42.41 ± 5.66^{a}	49.12 ± 7.88^{b}
Total lipids	20.06 ± 0.95	19.79 ± 3.16	17.44 ± 2.97	17.38 ± 1.93	21.22 ± 3.07	19.88 ± 1.71

(%)						
FAME (mg/g)	98.19 ± 6.58	101.36 ± 22.51	72.41 ± 3.94	60.36 ± 15.85[a]	93.77 ± 3.03[b]	98.16 ± 15.29[b]

Groups termed as "Total" include some components not shown among the selected fatty acids presented in the table. MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. Total lipids (%): percentage of lipids with respect to the total dry weight of the sample analyzed. FAME: Fatty acid methyl ester.

5.3.3.2. S. senegalensis

Similarly to what it has been found for *S. aurata*, the total lipid content of heads from *S. senegalensis* fed Diets 1, 2 and 3 were not significantly different. Senegalese sole fed Diet 1 showed a higher amount of 20:5n-3 and 22:5n-3 than fish fed Diets 2 and 3 at both 15 and 30 d sampling points (Table 5.5). Significant differences in 22:6n-3 and total n-3 PUFA content were observed at 15 d, with *S. senegalensis* fed Diet 1 showing a higher amount of these FA compared to post-larvae fed Diet 3. However, such significant differences were not observed at 30 d of feeding (Table 5.5). Sole fed Diet 1 for 30 d showed lower levels of 18:2n-6, 20:2n-6 and total n-6 PUFA in comparison to fish fed Diets 2 and 3.

Table 5.5. Selected fatty acid content (% of total fatty acids) of the total lipids from the head of *S. senegalensis* post-larvae fed experimental diets, with different PUFA content, during 15 and 30 d. Results are expressed as mean ± SD (n = 4). Different superscripts denote significant differences among diets for each sampling point (one way-ANOVA and Tukey test, $P \leq 0.05$).

	S. senegalensis					
	15 d			**30 d**		
Fatty acid	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 1**	**Diet 2**	**Diet 3**
18:2n-6	6.74 ± 1.99	11.15 ± 4.91	12.74 ± 5.55	7.33 ± 2.12^{a}	11.74 ± 3.11^{ab}	15.10 ± 4.58^{b}
20:2n-6	0.26 ± 0.06^{a}	0.54 ± 0.11^{ab}	0.80 ± 0.19^{b}	0.30 ± 0.06^{a}	0.57 ± 0.09^{b}	0.85 ± 0.17^{c}
20:4n-6	2.15 ± 0.16	2.14 ± 0.44	2.01 ± 0.32	1.77 ± 0.45	1.82 ± 0.27	1.65 ± 0.34
22:5n-6	0.92 ± 0.14	1.28 ± 0.24	1.10 ± 0.25	0.66 ± 0.17	1.12 ± 0.79	0.83 ± 0.20
18:3n-3	0.64 ± 0.26	0.80 ± 0.39	0.74 ± 0.26	0.62 ± 0.26	0.53 ± 0.18	0.63 ± 0.24
20:3n-3	0.17 ± 0.01^{a}	0.31 ± 0.04^{b}	0.31 ± 0.05^{b}	0.13 ± 0.02^{a}	0.18 ± 0.02^{b}	0.25 ± 0.00^{c}
20:4n-3	0.37 ± 0.20	0.18 ± 0.05	0.28 ± 0.00	0.24 ± 0.10	-	-
20:5n-3	2.53 ± 0.75^{b}	0.92 ± 0.31^{a}	0.78 ± 0.16^{a}	2.47 ± 0.76^{b}	0.88 ± 0.23^{a}	0.66 ± 0.03^{a}
22:5n-3	2.40 ± 0.41^{b}	1.27 ± 0.49^{a}	1.01 ± 0.29^{a}	2.39 ± 0.53^{b}	1.26 ± 0.30^{a}	1.00 ± 0.09^{a}
22:6n-3	19.44 ± 1.17^{b}	17.07 ± 1.37^{ab}	16.21 ± 1.37^{a}	18.95 ± 3.50	17.21 ± 1.11	17.65 ± 2.89
Total n-3 PUFA	25.56 ± 2.80^{c}	20.54 ± 2.65^{b}	19.33 ± 2.13^{a}	24.80 ± 5.17	20.07 ± 1.84	20.18 ± 3.24
Total n-6 PUFA	10.08 ± 2.19	15.10 ± 5.70	16.65 ± 6.31	10.06 ± 2.79^{a}	15.26 ± 4.26^{b}	18.43 ± 5.30^{b}
Total unsaturates	59.38 ± 9.40	57.45 ± 10.93	60.14 ± 11.86	60.95 ± 12.27	55.86 ± 8.30	59.27 ± 10.78
Total saturates	40.03 ± 7.83	2.40 ± 7.68	43.01 ± 8.81	37.14 ± 4.97^{a}	43.08 ± 4.86^{b}	40.31 ± 3.76^{b}
Total MUFA	22.40 ± 3.72	20.55 ± 1.91	20.40 ± 2.16	24.49 ± 3.88^{b}	19.72 ± 1.93^{a}	19.54 ± 1.90^{a}
Total PUFA	36.97 ± 5.68	36.90 ± 9.02	39.73 ± 9.70	36.46 ± 8.39	36.14 ± 6.36	39.73 ± 8.88
Total lipids (%)	16.10 ± 1.05	14.86 ± 3.28	15.15 ± 1.66	14.34 ± 2.86	12.64 ± 1.47	13.99 ± 2.76
FAME (mg/g)	49.35 ± 6.58	51.97 ± 3.47	47.79 ± 4.42	47.79 ± 4.42	46.66 ± 2.92	56.44 ± 13.21

Groups termed as "Total" include some components not shown among the selected fatty acids presented in the table. MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; (-): not detected. Total lipids (%): percentage of lipids with respect to the total dry weight of the sample analyzed. FAME: Fatty acid methyl ester.

5.3.4. Nutritional regulation

5.3.4.1. S. aurata

At 15 d of feeding, *fads2* showed an up-regulation in viscera (body part with the highest expression levels) of fish fed Diet 3 compared to fish fed Diets 1 and 2 (**Figure 5.4**). Similarly, *elovl5* presented an expression pattern characterized by an up-regulation in viscera, muscle and head of fish fed Diet 3 (**Figure 5.4**). Head showed the highest expression levels for *elovl4a* and *elovl4b* (**Figure 5.4**), but only *elovl4a* presented an up-regulation in fish fed Diet 2 in comparison to fish fed Diets 1 and 3 (**Figure 5.4**). At 30 d, *fads2* showed a similar expression pattern than that found at 15 d, being up-regulated in viscera of fish fed Diet 3 (**Figure 5.5**). *Elovl5* expression pattern was characterized by an up-regulation in viscera and head (body parts with the highest expression levels) of fish fed Diet 3 (**Figure 5.5**). Head presented the highest expression levels for *elovl4a* and *elovl4b* but, unlike results at 15 d of feeding, both *elovl4* genes were up-regulated in fish fed Diet 3 (**Figure 5.5**).

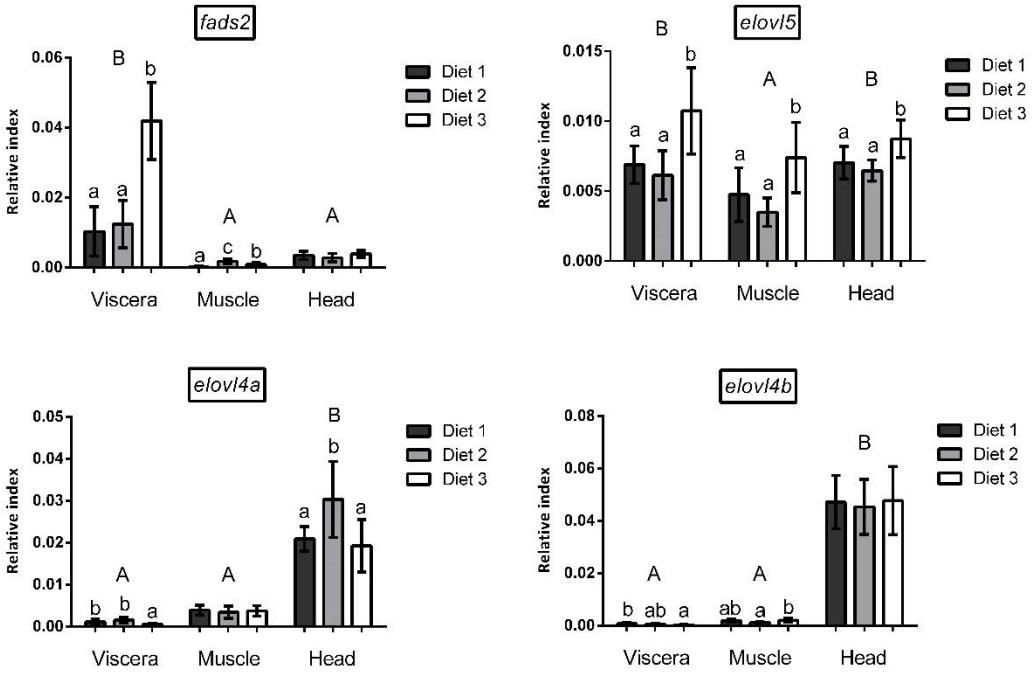

Figure 5.4. Expression pattern of *fads2*, *elovl5*, *elovl4a* and *elovl4b* in *S. aurata* post-larvae fed experimental diets (Diet 1, Diet 2 and Diet 3) during 15 d. The results showed as relative index, are β-actin normalized values (gene copy number / *β-actin* copy number) corresponding to the mean and standard deviation as error bars (n = 12). Lowercase letters above the columns denote significant differences (one way-ANOVA and Tukey test, $P \leq 0.05$) among dietary treatments for the same body part (viscera, muscle or head). Capital letters show significant differences (one way-ANOVA and Tukey test, $P \leq 0.05$) among body parts (three diets pooled).

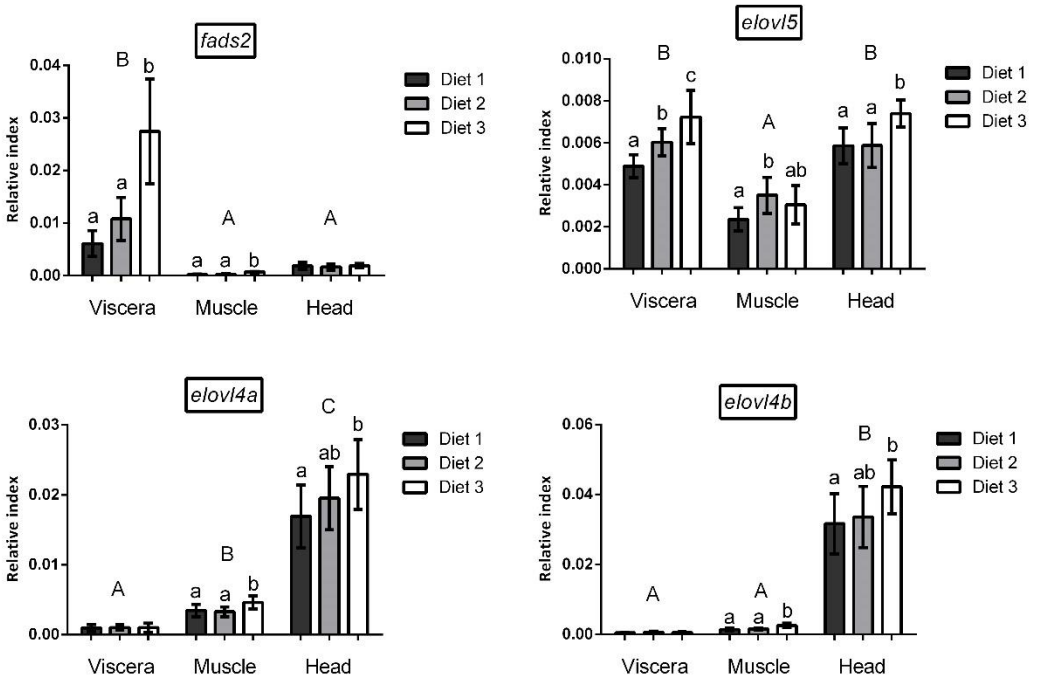

Figure 5.5. Expression pattern of *fads2*, *elovl5*, *elovl4a* and *elovl4b* in *S. aurata* post-larvae fed experimental diets (Diet 1, Diet 2 and Diet 3) during 30 d. The results showed as relative index, are β-actin normalized values (gene copy number / *β-actin* copy number) corresponding to the mean and standard deviation as error bars (n = 12). Lowercase letters above the columns denote significant differences (one way-ANOVA and Tukey test, $P \leq 0.05$) among dietary treatments for the same body part (viscera, muscle or head). Capital letters show significant differences (one way-ANOVA and Tukey test, $P \leq 0.05$) among body parts (three diets pooled).

5.3.4.2. S. senegalensis

Remarkably, at 15 d, *fads2* and *elovl5* showed an up-regulation in viscera of fish fed Diet 1 (**Figure 5.6**) compared to fish fed Diets 2 and 3. Head presented the highest expression levels for *elovl4a* and *elovl4b*, but no significant differences in their expression were found associated to diet (**Figure 5.6**). At 30 d of feeding, *fads2* was up-regulated in head of fish fed Diet 3, while no dietary effects were found in viscera and muscle (**Figure 5.7**). *Elovl5* showed an up-regulation in viscera (body part with the highest expression levels) and muscle of fish fed Diets 1 and 2, while *elovl5* was up-regulated in head of fish fed Diet 3 (**Figure 5.7**). Head presented the highest expression levels for *elovl4a* and *elovl4b*. Interestingly, both *elovl4* isoforms were nutritionally regulated in head (**Figure 5.7**). *Elovl4a* showed significant differences among the three diets, peaking in fish fed Diet 3, whereas *elovl4b* was up-regulated in fish fed Diets 1 and 2, between which no significant differences were detected.

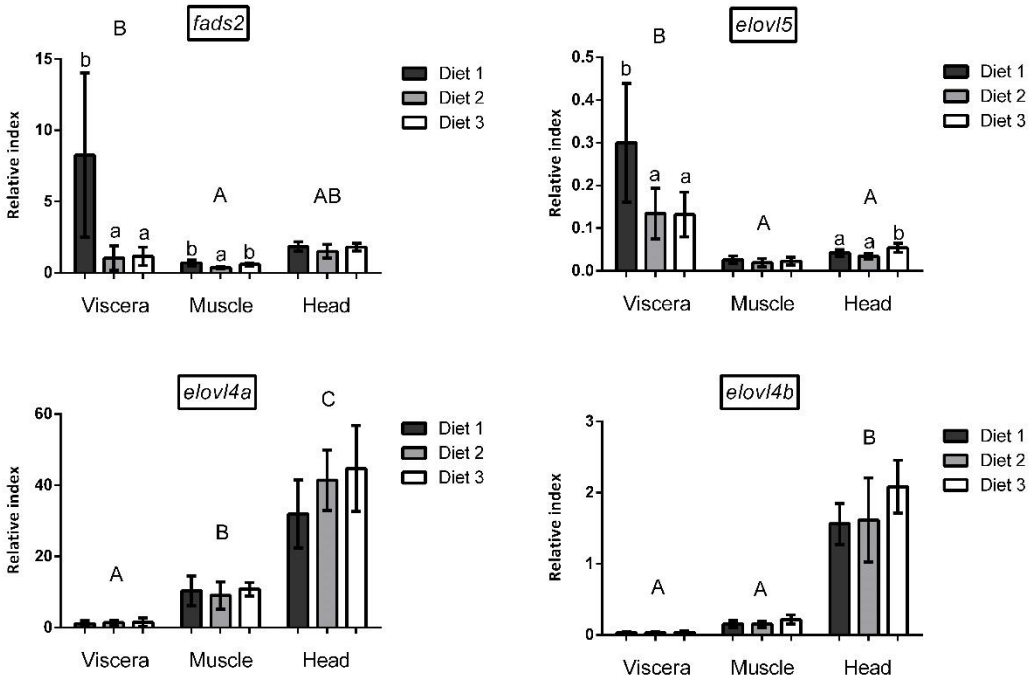

Figure 5.6. Expression pattern of *fads2*, *elovl5*, *elovl4a* and *elovl4b* in *S. senegalensis* post-larvae fed experimental diets (Diet 1, Diet 2 and Diet 3) during 15 d. The results showed as relative index, are β-actin normalized values (gene copy number / *β-actin* copy number) corresponding to the mean and standard deviation as error bars (n = 12). Lowercase letters above the columns denote significant differences (one way-ANOVA and Tukey test, $P \leq 0.05$) among dietary treatments for the same body part (viscera, muscle or head). Capital letters show significant differences (one way-ANOVA and Tukey test, $P \leq 0.05$) among body parts (three diets pooled).

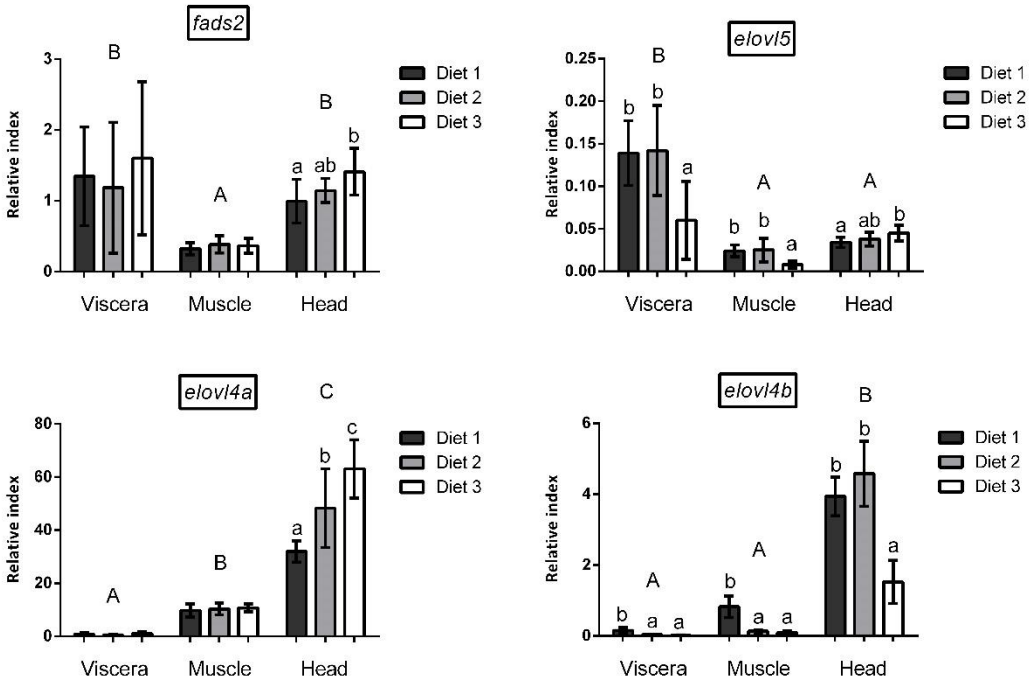

Figure 5.7. Expression pattern of *fads2*, *elovl5*, *elovl4a* and *elovl4b* in *S. senegalensis* post-larvae fed experimental diets (Diet 1, Diet 2 and Diet 3) during 30 d. The results showed as relative index, are β-actin normalized values (gene copy number / *β-actin* copy number) corresponding to the mean and standard deviation as error bars (n = 12). Lowercase letters above the columns denote significant differences (one way-ANOVA and Tukey test, $P \leq 0.05$) among dietary treatments for the same body parts (viscera, muscle or head). Capital letters show significant differences (one way-ANOVA and Tukey test, $P \leq 0.05$) among body parts (three diets pooled).

5.4. Discussion

It is well known that an adequate supply of LC-PUFA is critical for optimal growth and survival of early stages of fish (Glencross, 2009; Hamre *et al*., 2013; Tocher, 2010). Our survival results are in agreement with those described by other authors for early stages of *S. aurata* (Izquierdo *et al*., 2008; Koven *et al*., 1990) and *S. senegalensis* (Morais *et al*., 2004; Villalta *et al*., 2005), since no adverse effects were found in final survival rates associated to dietary LC-PUFA content for both species. Regarding the biometric parameters, the post-larvae growth differences observed in our study at 30 d are in agreement with those observed by other authors in early stages of *S. aurata* (Mourente *et al*., 1993; Salhi *et al*., 1999; Turkmen *et al*., 2017) and *S. senegalensis* (Bonacic *et al*., 2016; Pinto *et al*., 2016), in which increases in dietary n-3 LC-PUFA contents promoted fish growth.

Our results suggest that diet can influence the FA profiles of the head lipids in both species. Interestingly, in *S. senegalensis* fed during 30 d, no significant differences were observed in DHA levels, regardless of the DHA contents of the diet. This is in agreement with the results reported by Morais *et al*. (2004) and Navarro-Guillén *et al*. (2014), who found that *S. senegalensis* larvae fed a DHA deficient diet showed higher DHA levels than those fed diets richer in DHA. While the specific mechanism is not fully understood, this is likely due to the presence of a Fads2 with Δ4 desaturase activity in *S. senegalensis* (Morais *et al*., 2012), which

enables DHA biosynthesis from EPA through the so-called "Δ4 pathway" (Li *et al*., 2010). This pathway has been regarded as a more direct route for production of DHA, involving one single elongation of EPA to synthesize 22:5n-3 and a Δ4 desaturation to DHA in comparison to the "Δ6 pathway" (Li *et al*., 2010; Oboh *et al*., 2017a). Indeed, the Δ6 pathway, apparently operative in *S. aurata* (Seiliez *et al*., 2003), requires two consecutive elongations from EPA to produce 24:5n-3, a Δ6 desaturation to 24:6n-3 mediated by Fads2, and a peroxisomal β-oxidation to DHA (Sprecher, 2000). Thus, the abovementioned absence of significant differences in DHA contents of head lipids from *S. senegalensis* fed during 30 d, along with the up-regulation of *fads2* observed in the head of *S. senegalensis* post-larvae fed during 30 d with Diet 3 (low DHA) could be indicative of the importance of DHA production via the Δ4 desaturation pathway in the Senegalese sole, satisfying the high requirements of this compound in neural tissues (Mourente, 2003; Tocher and Harvie, 1988) and ensuring optimal DHA levels even under limited dietary supply (Kuah *et al*., 2015; Morais *et al*., 2012). Moreover, the FA profile denoted a relation between the dietary 18:2n-6 content and its levels in the heads of both species. So, while this result would indicate accumulation/retention of 18:2n-6 in head lipids, it is also interesting to note that some metabolic products from 18:2n-6 are also significantly increased in both fish species. In seabream, significantly higher contents of 18:3n-6 (Δ6 desaturation product), 20:2n-6 (elongation product) and

20:3n-6 (Δ8 desaturation product from 20:2n-6) were observed in fish fed Diet 3 compared to fish fed Diet 1 (FO rich). In Senegalese sole, significantly higher contents of 20:2n-6 (elongation product of 18:2n-6) were detected when comparing FA from heads of fish fed Diets 1 and 3. These results are consistent with the desaturase and elongase capacities described for both species. Whereas both species possess Elovl5 with the ability to elongate 18:2n-6, only seabream has a Fads2 with Δ6 and Δ8 desaturase capacity (Monroig *et al*., 2011b). So, while a fraction of the dietary 18:2n-6 is retained unmodified in the head lipids, part is also bioconverted by endogenous enzymes existing in both species (Morais *et al*., 2012, Zheng *et al*., 2004). It is also likely that, at least in the case of seabream, competition between Fads2 and Elovl5 might have also existed since FA such as 18:2n-6 are utilized by both enzymes.

Related to the *S. senegalensis* visceral *fads2*, an up-regulation was shown in fish fed the richest diet in LC-PUFA (Diet 1) after 15 d. These results are opposed to those observed in *S. aurata* visceral *fads2*, since in this case, it was up-regulated in response to a deficient LC-PUFA dietary supply, i.e. in fish fed Diet 3, similarly to what was described in seabream larvae fed microdiets during 17 d by Izquierdo *et al*. (2008). This opposite effect can be due to multiple factors as the species-specific desaturase activity showed by both fish Fads2, the different physiological age of both fish employed in the assay, or the possible differences in the amount of food

ingested by both species during the first trial period, and therefore, the amount of dietary PUFA substrate available for Fads2 enzyme activity. However, the high dispersion of values showed for *fads2* in viscera of *S. senegalensis* fed Diet 1 point to the need of further analyses to confirm the relationship between high dietary PUFA content and the *fads2* up-regulation in visceral tissues of Senegalese sole post-larvae. Interestingly, at 30 d no significant differences among dietary treatments were observed in the visceral *S. senegalensis fads2* expression values. This could be indicative that, even though additional assays are necessary to confirm this question, the visceral *fads2* expression could be time-dependent.

Along that of *fads2*, nutritional regulation of *elovl5* was also observed in our study. Functional characterization assays have denoted that fish Elovl5 show a versatile elongation activity towards C_{18} (18:4n-3 and 18:3n-6), C_{20} (20:4n-3 and 20:3n-6) and C_{22} (22:5n-3 and 22:4n-6) PUFA (Agaba *et al*., 2005), but also to elongate monounsaturated FA (Kabeya *et al*., 2015; Monroig *et al*., 2013). As described in other carnivorous teleost species such as juveniles of both *Acanthopagrus schlegelii* (Jin *et al*., 2017a) and *Nibea coibor* (Lin *et al*., 2018b) fed diets with different FA profiles during 2 months, the *S. aurata elovl5* was up-regulated in viscera, muscle and head of fish fed low LC-PUFA diet (Diet 3) showing a preponderant role to elongate n-6 PUFA, 18:3n-6 and 20:3n-6. In the case of *S. senegalensis*, viscera showed the highest *elovl5* expression values but

remarkably, *elovl5* was up-regulated in fish fed highest n-3 PUFA diet (Diet 1). So, head FA profile of fish fed Diet 1 showed highest 22:5n-3 levels (probably mobilized from the liver) in agreement with the high 22:5n-3 elongation activity described in *S. senegalensis elovl5* by Morais *et al*. (2012). Interestingly, a similar expression pattern for *elovl5* was shown in liver and brain of *Channa striata* juveniles (fed during 2 months), another carnivorous teleost that, like *S. senegalensis*, possess a Δ4 Fads2 (Kuah *et al*., 2015) although, unlike *S. senegalensis*, has a second Δ6/Δ5 Fads2 (Kuah *et al*., 2016). Thus, the differences shown between the expression pattern of *S. aurata* Δ6 and *S. senegalensis* Δ4 *fads2* in head, as well as in the visceral *elovl5* regulation, as response to a reduction of the LC-PUFA dietary supply, could be an evidence of the species-specific functionality of *elovl5* and the preponderant role of Δ4 pathway in the DHA biosynthesis under precursor limitation.

For both species, mRNA transcripts of *elovl4* genes, responsible for VLC-PUFA biosynthesis, were detected in the three body parts analyzed, although both isoforms were strongly and preferentially expressed in the head. Such tissue expression, previously reported in other fish species (Betancor *et al*., 2020; Li *et al*., 2017a, 2017b; Yan *et al*., 2018; Zhao *et al*., 2019), is likely associated to the important biological functions that Elovl4 enzymes play in biosynthesis of VLC-PUFA for neural tissues (Agbaga *et al*., 2010; Deák *et al*., 2019; Dyall, 2015; Xue *et*

al., 2014). It is important to note that, along their well-established role in VLC-PUFA biosynthesis, teleost fish Elovl4 have been also suggested to contribute to DHA biosynthesis via the Sprecher pathway, since they can elongate 20:5n-3 (EPA) and 22:5n-3 to 24:5n-3 (Oboh *et al*., 2017a; Sprecher, 2000). Such elongation capacity of teleost Elovl4, which could partly compensate the absence of *elovl2* from genomes of most of the marine fish species (Monroig *et al*., 2011a, 2018), has been recently demonstrated in the Atlantic bluefin tuna (*Thunnus thynnus*) (Betancor *et al*., 2020). Collectively, due to the key roles that the biosynthesis products of Elovl4 (i.e. VLC-PUFA, DHA) play in the correct brain and retina development and functionality, *elovl4* is considered a crucial gene in neuronal function of vertebrates including fish (Agbaga *et al*., 2010; Deák *et al*., 2019; Monroig *et al*., 2011; Sherry *et al*., 2017).

Our results on nutritional regulation of *elovl4* are in agreement with those reported in other aquatic organisms (Li *et al*., 2017a, 2017b; Lin *et al*., 2018a; Yan *et al*., 2018; Zhao *et al*., 2019) where a dietary LC-PUFA reduction resulted in an up-regulation of *elovl4* genes. More specifically, both *elovl4a* and *elovl4b* from *S. aurata* and *elovl4a* from *S. senegalensis* were up-regulated in post-larvae fed Diet 3 for 30 d, which contained the lowest LC-PUFA levels among the diets used in the present study. This is indicative that a deficient dietary LC-PUFA content could increase the *elovl4* gene transcription to compensate the lack of VLC-PUFA

substrates, i.e. LC-PUFA, in both species. Unlike other *elovl4* genes studied herein, the *S. senegalensis elovl4b* was down-regulated in fish fed low LC-PUFA diet as reported for the Atlantic bluefin tuna *elovl4b* (Betancor *et al*., 2020). Why both *S. senegalensis elovl4a* and *elovl4b* responded differently to a low LC-PUFA diet, whereas both *elovl4* isoforms had a consistent response to the same dietary condition in seabream, is a question that requires further investigation. However, the apparently different *elovl4* regulation of both species can be accounted for hypothetically different VLC-PUFA requirements as suggested in a previous study (Torres *et al*., 2020). In agreement with the results here exposed, previous *elovl4* nutritional regulation assays (Torres *et al*., 2020), performed in earlier stages, i.e. not weaned larvae, of *S. aurata* and *S. senegalensis*, suggest that *S. senegalensis elovl4b* could be regulated positively when fed high LC-PUFA dietary content during early life-cycle stages. However, the results presented in Torres *et al.* (2020) and those found in the present study show some differences in the nutritional regulation of both *elovl4* in *S. aurata* and *elovl4a* in *S. senegalensis* according to the VLC-PUFA putative needs associated with each life-stage and the LC-PUFA dietary availability.

To conclude, our results showed that dietary LC-PUFA content, although without apparent effect in survival, determined growth performance of *S. aurata* and *S. senegalensis* post-larvae. Moreover, the expression pattern of genes involved in the biosynthesis of VLC-PUFA (*elovl4a*, *elovl4b*) and their precursors, i.e. LC-

PUFA, (*fads2*, *elovl5*) can be regulated by dietary LC-PUFA content. In addition, our results establish that the high expression of both *elovl4* isoforms detected in the head of gilthead seabream and Senegalese sole post-larvae can be associated to high VLC-PUFA requirements necessary for optimal development and functionality of neural tissues. These results can provide insight into aquafeed formulation for first stages of farmed fish, such as *S. aurata* and *S. senegalensis*, opening the possibility to incorporate successfully alternative lipid sources, through an early nutritional programming that stimulates the VLC-PUFA biosynthesis during the initial exogenous feeding stages.

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CAPÍTULO 6:

Discusión general

Los ácidos grasos, en especial los ácidos grasos de cadena larga (C_{20-24}) o LC-PUFA (en inglés *long-chain polyunsaturated fatty acids*), poseen un papel fundamental en numerosos procesos fisiológicos y metabólicos de vital importancia en vertebrados (Castro *et al*., 2016; Schmitz y Ecker, 2008). Así, los organismos marinos, especialmente durante las primeras etapas de su ciclo de vida (fase larvaria/poslarvaria), son especialmente sensibles a la baja disponibilidad de ciertos LC-PUFA, como son el ácido araquidónico (ARA; 20:4n-6), el ácido eicosapentaenoico (EPA; 20:5n-3) y el ácido docosahexaenoico (DHA; 22:6n-3) (Sargent *et al*., 1999; Tocher, 2010, 2015). Estos compuestos son considerados esenciales (en inglés *essential fatty acids*; EFA) para la mayoría de organismos marinos cultivados, pues, de forma generalizada, no pueden ser sintetizados de manera endógena en cantidades suficientes para satisfacer correctamente sus necesidades fisiológicas. En condiciones naturales, estos requerimientos son cubiertos debido al acceso a una dieta más variada y rica en EPA y DHA (Sargent *et al*., 2003). Consecuentemente, durante su evolución, las especies marinas de hábitos carnívoros han perdido la capacidad de bioconvertir de manera eficiente su ingesta dietética muy limitada de 18:3n-3 a 20:5n-3 y 22:6n-3 (Castro *et al*., 2016; Sargent *et al*., 2003). Por lo tanto, el uso en acuicultura de dietas subóptimas en LC-PUFA n-3, puede conllevar efectos perjudiciales para el desarrollo de los teleóteos marinos cultivados (Tocher, 2015).

Los requerimientos de LC-PUFA varían, cualitativa y cuantitativamente, en función de la especie, su nivel trófico, la fase del desarrollo y el medio ambiente (Sargent *et al*., 2003; Tocher, 2010). Debido al rápido desarrollo y crecimiento tisular, especialmente de los tejidos neurales donde estos compuestos son acumulados en altas cantidades (Hamre *et al*., 2013; Izquierdo *et al*., 2015), es de vital importancia asegurar un buen aporte dietario de EFA durante las etapas iniciales del desarrollo de los peces (Sargent *et al*., 2003; Tocher, 2010). En base a esto, y teniendo en cuenta aspectos como el pequeño tamaño de las larvas y la presencia de un sistema digestivo sin desarrollar, la primera fase de cría denominada *hatchery* o *nursery* es considerada una etapa crítica en el cultivo de especies marinas (Monroig *et al*., 2018; Sargent *et al*., 1997; Tocher, 2010, 2015).

Teniendo en cuenta la esencialidad y, por ende, los altos requerimientos nutricionales de ácidos grasos como el ARA, el EPA y/o el DHA durante el cultivo de especies acuícolas, así como que el pescado representa la principal fuente de LC-PUFA n-3 para el ser humano (Larsen *et al*., 2011; Strobel *et al*., 2012), se ha prestado especial atención al estudio del mecanismo biosintético de los LC-PUFA y su regulación metabólica en peces con fines aplicados a la acuicultura. En consecuencia, existe un interés creciente por el estudio de las rutas de biosíntesis de estos compuestos en las especies marinas cultivadas. Asimismo, son cada vez más los trabajos centrados en analizar la influencia de las "futuras dietas", formuladas

primando la sustitución de compuestos de origen animal (harina y aceite de pescado) por ingredientes de origen vegetal (Miller *et al*., 2008; Turchini *et al*., 2011), sobre el metabolismo biosintético de los LC-PUFA durante el desarrollo de los peces marinos cultivados (Benedito-Palos *et al*., 2008; Geay *et al*., 2010; Martins *et al*., 2012; Navarro-Guillén *et al*., 2014).

Por otra parte, a pesar de las múltiples evidencias que resaltan la importancia de los ácidos grasos de cadena muy larga (>C_{24}) o VLC-FA (en inglés *very long-chain fatty acids*) en importantes procesos biológicos como la visión, la función cerebral, la permeabilidad de la piel y la reproducción en mamíferos (Agbaga *et al*., 2008; Aldahmesh *et al*., 2011; Furland *et al*., 2007; Mandal *et al*., 2004; Poulos, 1995), su estudio en organismos acuáticos, especialmente en aquellas especies de interés acuícola, ha sido casi inexistente. Estos compuestos, aunque siempre presentes en muy pequeñas cantidades, podrían ejercer un papel clave en el correcto desarrollo y funcionalidad del tejido nervioso durante las etapas iniciales del desarrollo de los teleósteos, ya que, tanto los ácidos grasos saturados de cadena muy larga (en inglés *very long-chain saturated fatty acids*; VLC-SFA), como los ácidos grasos poliinsaturados de cadena muy larga (en inglés *very long-chain polyunsaturated fatty acids*; VLC-PUFA) participan activamente en la protección de los fotorreceptores (Bennett *et al*., 2014) y en el proceso de neurotransmisión llevado a cabo en el sistema nervioso de los vertebrados (Deák *et al*., 2019). Por lo tanto, una

inadecuada biosíntesis de estos compuestos podría causar daños a nivel visual y/o neuronal, perjudicando al sistema cognitivo de los peces y por tanto a su desarrollo, crecimiento y supervivencia.

En este contexto, como se enfatiza a lo largo de este trabajo de investigación, se considera de vital importancia el estudio, la caracterización y el análisis de la regulación nutricional, a nivel de especie, del equipamiento enzimático implicado en la síntesis endógena de los VLC-PUFA, y de los genes que lo codifican. Estos ácidos grasos son biosintetizados por dos isoformas enzimáticas denominadas elongasas 4 de ácidos grasos de cadena muy larga, en inglés *elongation of very long chain fatty acid* (Elovl) *4 proteins a* (Elovl4a) y *b* (Elovl4b). Además, para una mejor comprensión de las rutas anabólicas de los VLC-PUFA, se han de tener en cuenta los genes que codifican para las enzimas que participan en la síntesis de sus precursores, los LC-PUFA. Así, en su síntesis endógena a partir de C_{18} PUFA (ácido α-linolénico; ALA, 18:3n-3 y ácido linoleico; LA, 18:2n-6), participan la enzima desaturasa 2 de ácidos grasos, en inglés *fatty acyl desaturase* 2 (Fads2) y la elongasa 5, en inglés *elongation of very long chain fatty acid 5 protein* (Elovl5). En base a ello, la información recogida en los capítulos precedentes es crucial para comprender la capacidad que una especie dada tiene para la producción endógena de estos nutrientes, que son de vital importancia especialmente durante su desarrollo temprano.

Estudios previos llevados a cabo en lenguado senegalés (*Solea senegalensis*) y en dorada (*Sparus aurata*) confirmaron que, si bien ambas especies pueden sintetizar enzimas Elovl5 funcionalmente equivalentes, difieren en la función de sus enzimas Fads2 (Agaba *et al*., 2005; Morais *et al*., 2012). Por un lado, *S. aurata* tiene una Fads2 con actividad desaturasa Δ6 (Seiliez *et al*., 2003; Zheng *et al*., 2004), mientras que por otro lado, *S. senegalensis* tiene una Fads2 con actividad desaturasa Δ4 (Morais *et al*., 2012). Esta diferencia determina su capacidad para biosintetizar DHA, bien a través de una ruta más directa, conocida como "vía Δ4" llevada a cabo en *S. senegalensis* (Morais *et al*., 2012) o a través de otra ruta más compleja, conocida como "vía de Sprecher", llevada a cabo en *S. aurata* (Oboh *et al*., 2017a; Sprecher, 2000) (**Figura 3.1**).

Al mismo tiempo, aunque teóricamente todos los teleósteos poseen dos isoformas de *elovl4*, denominadas *elovl4a* y *elovl4b* según la homología con sus ortólogos en *Danio rerio* (Monroig *et al*., 2010), la presencia de ambas isoformas no ha sido confirmada en muchas de las especies de peces cultivados objeto de estudio (Betancor *et al*., 2019; Carmona-Antoñanzas *et al*., 2011; Kabeya *et al*., 2015; Monroig *et al*., 2011; Zhao *et al*., 2019). Sin embargo, en consonancia con lo observado en varias especies de teleósteos, como p. ej. *Danio rerio* (Monroig *et al*., 2010), *Misgurnus anguillicaudatus* (Yan *et al*., 2018), *Acanthopagrus schlegelii* (Jin *et al*., 2017) y *Colossoma macropomum* (Ferraz *et al*., 2020), nuestro estudio

determinó que tanto *elovl4a*, como *elovl4b* están presentes en *S. aurata* y en *S. senegalensis* y que las proteínas que codifican son funcionales (Capítulo 3; Morais *et al*., 2020).

Además, el análisis de secuencias y el filogenético (Capítulo 3; Morais *et al*., 2020) ayudan a evidenciar una probable especialización funcional de estas proteínas en peces (Monroig *et al*., 2011; Zhao *et al*., 2019). Dicha especialización está marcada por la presencia y conservación de ambas isoformas de *elovl4* en el genoma (Castro *et al*., 2016; Jin *et al*., 2017; Kabeya *et al*., 2015; Monroig *et al*., 2010; Oboh *et al*., 2017b; Yan *et al*., 2018), así como por su clara segregación en grupos separados (*clusters*) (**Figura 3.3**). De igual manera, el análisis de los resultados procedentes de la caracterización funcional de las dos isoformas de Elovl4 en estas dos especies de teleósteos con historias vitales distintas, hábitos alimenticios y mecanismos de biosíntesis de LC-PUFA notablemente diferentes (Morais *et al*., 2012; Seiliez *et al*., 2003; Zheng *et al*., 2004), ayuda a poner de manifiesto dicha especialización.

Así, aunque los resultados confirman que las dos isoformas de Elovl4 participan en la biosíntesis de VLC-SFA y VLC-PUFA, tanto en dorada como en lenguado, se observaron diferencias en su capacidad de elongación a nivel intra- e interespecífico. Se puso de manifiesto que, como se observó en *D. rerio* (Monroig *et al*., 2010), Elovl4a parece estar más involucrada en la biosíntesis de VLC-SFA que

Elovl4b en las dos especies, siendo esta apreciación más evidente en la dorada. Además, se advirtieron algunas diferencias relacionadas con la afinidad que muestran hacia los ácidos grasos que actúan como sustratos de elongación para ambas enzimas. Así, en la dorada, Elovl4a y Elovl4b mostraron una mayor afinidad por el 26:0, mientras que los ácidos grasos saturados ≤C_{24} parecen ser más adecuados para las dos isoformas de Elovl4 en el lenguado. De igual manera, se observaron diferencias específicas en la capacidad de elongación de los VLC-PUFA para ambas isoformas, pues tanto en dorada como en lenguado, la Elovl4a mostró una mayor capacidad de elongación para los sustratos PUFA n-6, mientras que la Elovl4b mostró una mayor afinidad hacia los sustratos PUFA n-3. Estas diferencias, en cuanto a la preferencia de sustratos, fueron menos claras en el caso del lenguado. Aun así, solo la Elovl4b pudo elongar el DHA, hasta 32:6n-3 en ambas especies.

Estas diferencias en la capacidad de síntesis de los VLC-SFA y los VLC-PUFA, podrían estar relacionadas con los hipotéticos diferentes requerimientos de VLC-FA para las dos especies de teleósteos, especialmente durante las primeras etapas del desarrollo, cuando su deficiencia puede alterar/dañar su sistema visual y/o neuronal, todavía en formación. Además, como se ha demostrado en un ensayo llevado a cabo en peces cebra (*D. rerio*) cuyo gen *elovl2* fue inactivado (Sun *et al*., 2020), la actividad de la enzima Elovl2 es determinante en la síntesis endógena de DHA. En base a ello, es probable que la conservación de ambas isoformas de Elovl4

con capacidad para elongar el 22:5n-3 hasta 24:5n-3 pueda conferir una ventaja adaptativa sustancial en especies de peces marinos que han perdido el gen *elovl2* durante su evolución (Castro *et al*., 2016; Jin *et al*., 2017; Monroig *et al*., 2018).

Teniendo esto en mente, podemos relacionar el patrón de expresión temporal de los genes *elovl* y *fads* obtenido durante el desarrollo ontogénico de ambas especies (Capítulo 4; Torres *et al*., 2020a), con las elevadas necesidades fisiológicas de LC-PUFA y VLC-PUFA asosociadas al desarrollo larvario temprano de los teleósteos marinos. De esta manera, los resultados de expresión de los genes *fads2*, *elovl5*, *elovl4a* y *elovl4b* revelaron la existencia de diferencias, a nivel inter- e intraespecífico, en el patrón de expresión temporal, que se caracterizó por un incremento diferencial en la pauta de expresión de ambas isoformas de *elovl4* en cada especie. Estos picos en la expresión de *elovl4a* y *elovl4b* parecen coincidir con el momento en el cual ocurren los eventos más relevantes durante la formación de la retina en las larvas de la dorada y el lenguado senegalés. El desarrollo del tejido visual no se produce sincrónicamente en ambas especies, puesto que, mientras que en el lenguado, la retina está completamente desarrollada y es funcional en torno a las 48 horas posteriores a la eclosión (Bejarano-Escobar *et al*., 2010), su desarrollo es más lento en la dorada (Pavón-Muñoz *et al*., 2016). Durante este proceso, en el que los peces experimentan grandes cambios morfológicos y fisiológicos, es importante que las larvas dispongan no solo de una buena reserva vitelina de

nutrientes, sino además, de un óptimo mecanismo de biosíntesis de ácidos grasos que les permita afrontar los grandes cambios que ocurren a lo largo del desarrollo ontogénico. Asimismo, hay que destacar la especial relevancia de una adecuada síntesis de ciertos LC-PUFA, como el DHA, así como de los VLC-PUFA, cuya correcta biodisponibilidad es vital para el buen funcionamiento de las membranas biológicas. Estos compuestos ejercen un papel clave en el desarrollo y correcta funcionalidad de las células del sistema inmune y del tejido neural, y por ende, son de vital importancia para el desarrollo visual y cognitivo de los peces durante su etapa inicial (Barabas *et al*., 2013; Bell y Tocher, 1989; Bell *et al*., 1995; Benitez-Santana *et al*., 2007; Mourente *et al*., 2003; Tocher y Harvie, 1988).

Tal como revelaron los resultados de caracterización funcional, tanto la Elov4a como la Elovl4b participan activamente en la síntesis de los VLC-PUFA en las dos especies, siendo la Elovl4b más eficiente sintetizando 22:5n-3 y 24:5n-3 a partir de 20:5n-3. Ambos compuestos, 22:5n-3 a través de la vía desaturasa Δ4, llevada a cabo por la Fads2 de *S. senegalensis*, y 24:5n-3 a través de la vía desaturasa Δ6, llevada a cabo por la Fads2 de *S. aurata*, actúan como precursores biosintéticos del DHA. Por esta razón, se sugiere que, la sincronía entre el momento en que se produce la retinogénesis en ambas especies y una mayor expresión de los genes *elovl4*, podría contribuir a explicar la importancia que tanto el DHA como los VLC-PUFA tienen para la correcta formación de las estructuras visuales durante las etapas iniciales del

desarrollo larvario en peces marinos. Además de la asincronía en el patrón de expresión temporal de *elovl4a* y *elovl4b* observada en las larvas de dorada y de lenguado senegalés, se puso de manifiesto la existencia de un patrón espécifico de expresión tisular de ambos genes en individuos adultos. Éste, al igual que lo observado en otras especies de teleósteos (Betancor *et al*., 2020; Carmona-Antoñanzas *et al*., 2011; Jin *et al*., 2017; Kabeya *et al*., 2015; Monroig *et al*., 2010; Zhao *et al*., 2019), se caracteriza por una expresión preferencial de *elovl4a* en el cerebro y de *elovl4b* en los ojos. Estas características observadas en el patrón de expresión espacio-temporal de ambos genes, unidas a las diferencias observadas a nivel de especificidad de sustrato y/o capacidad de elongación de las enzimas Elovl4a y Elovl4b (Capítulo 3; Morais *et al*., 2020), podrían evidenciar el papel vital que ambas isoformas, y por tanto, sus productos de biosíntesis, desempeñan en procesos fisiológicos cruciales, tales como la visión y la función cerebral en peces marinos. Por lo tanto, una correcta estimulación de la biosíntesis de los VLC-FA adquiere especial importancia durante el desarrollo temprano de los teleósteos, periodo en el que se llevan a cabo importantes cambios a nivel fisiológico (Zambonino-Infante y Cahu, 2001), y de cuyo buen desarrollo y función depende, en gran medida, el correcto crecimiento y la supervivencia larvaria.

Asimismo, este patrón de expresión podría verse modificado en función de los requirimientos específicos de VLC-PUFA asociados a cada etapa del desarrollo en

función de la especie, a las condiciones del cultivo larvario, al estado fisiológico de los peces o incluso al metabolismo lipídico de los progenitores (Turkmen *et al*., 2019). Además, podría ser que, tal como sugieren los altos niveles de transcripción de *elovl5* observados en huevos de *S. senegalensis* (Capítulo 4; Torres *et al*., 2020a), se produzca una transferencia materna de *elovl5* que permita iniciar de manera temprana la síntesis endógena de LC-PUFA en el embrión (Monroig *et al*., 2009), garantizando así la disponibilidad, no sólo de DHA, sino de sustratos para la síntesis endógena de VLC-PUFA.

Teniendo esto en cuenta, el estudio de la regulación nutricional de los genes involucrados en la biosíntesis, tanto de los LC-PUFA como de los VLC-PUFA, a lo largo de las diferentes fases del cultivo de especies marinas, es de vital importancia para garantizar la correcta disponibilidad de estos nutrientes en los alimentos que se utilizan, especialmente durante las primeras etapas del desarrollo. Por ello, la regulación nutricional de los genes *fads2* y *elovl5* ha sido ampliamente estudiada en diferentes especies de peces (Izquierdo *et al*., 2008; Kuah *et al*., 2015; Li *et al*., 2016, 2017; Morais *et al*., 2012; Turkmen *et al*., 2017, 2019). Sin embargo, a excepción de algunos trabajos llevados a cabo en teleósteos como *Larimichthys crocea* (Li *et al*., 2017a), *Oncorhynchus mykiss* (Zhao *et al*., 2019) y *Thunnus thynnus* (Betancor *et al*., 2020), existen pocos estudios que pongan el enfoque sobre la regulación nutricional de los genes *elovl4* en peces marinos cultivados.

Los resultados sobre la regulación nutricional de *fads2* y *elovl5* observados en estadios tempranos, es decir en larvas tempranas (*early larvae*) con 14 días poseclosión (dpe), y en larvas tardías con 40 dpe (*late larvae*) denotan la ausencia de efectos nutricionales en la expresión de dichos genes en *S. aurata* (Capítulo 4; Torres *et al*., 2020a). Estos resultados parecen contrastar con lo observado por Turkmen *et al*., (2017) en larvas de dorada donde, aunque no se observaron diferencias significativas entre los valores de expresión de *fads2* en larvas alimentadas con las dietas con el menor y el mayor contenido en LC-PUFA n-3, sí se observó cierta tendencia hacia el efecto de los niveles dietarios de LC-PUFA n-3 sobre la expresión de *fads2*. Estas diferencias observadas en los patrones de expresión de *fads2* pueden verse influidas por la naturaleza de las dietas (presas vivas *vs* dietas inertes), por el desigual contenido dietario en ácidos grasos, así como por la historia nutricional de los progenitores y la calidad de la puesta (Ferosekhan *et al*., 2020; Izquierdo *et al*., 2015; Turkmen *et al*., 2019, 2020). Curiosamente, nuestros resultados coinciden con lo observado en larvas tempranas (10 dpe) de lubina (*Dicentrarchus labrax*) (Vagner *et al*., 2009) cuya actividad desaturasa no se vio afectada por el contenido en PUFA presente en la dieta. Esto podría ser indicativo de que la Fads2 con actividad desaturasa Δ6 opera de forma insuficiente en la ruta de biosíntesis de los PUFA, para responder adecuadamente a los altos requerimientos de EPA y DHA necesarios durante las etapas más iniciales del desarrollo larvario de *S. aurata* (larvas tempranas

y tardías). Por lo tanto, éstos deben cubrirse con una dieta rica en LC-PUFA n-3. Esta situación parece cambiar a lo largo del desarrollo, pues, tal como parecen indicar los resultados de expresión de *fads2* y *elovl5* en poslarvas de dorada (Izquierdo *et al*., 2008; Martins *et al*., 2012) y lubina (Vagner *et al*., 2009), su actividad puede ser modulada, en cierta medida, a través de la dieta. De este modo, los resultados obtenidos para *fads2* en la región vísceral de poslarvas de *S. aurata* (Capítulo 5; Torres *et al*., 2020b) están en consonancia con los trabajos que indican que la actividad desaturasa Δ5/Δ6 en peces responde a los niveles dietarios de PUFA, aumentando la expresión de *fads2* para compensar un suministro deficiente de PUFA en la dieta (Izquierdo *et al*., 2008, 2015; Ren *et al*., 2012; Seiliez *et al*., 2003). Esta regulación positiva ayudaría a contrarrestar la aparente incapacidad que los peces marinos presentan para convertir el 18:3n-3 en EPA (20:5n-3) y DHA (22:6n-3). Sin embargo, la expresión de *fads2* en la cabeza no parece verse afectada por el contenido en ácidos grasos de la dieta. Esto podría ser indicativo de que el metabolismo de los LC-PUFA en los tejidos neurales atiende mayoritariamemante a criterios fisiológicos endógenos y, por lo tanto, existe una mínima influencia de la dieta en su biosíntesis (Xu *et al*., 2020). Además, este metabolismo podría verse influido por la dificultad que las larvas/poslarvas presentan para transportar los lípidos desde los enterocitos del tracto digestivo en desarrollo hasta el resto de tejidos corporales (Izquierdo *et al*., 2000). Así, cabe resaltar la importancia de una

correcta biosíntesis local de LC-PUFA, especialmente en tejidos cerebrales donde éstos tienen un papel fundamental para el correcto desarrollo y función del sistema nervioso (Xu *et al*., 2020).

Estos resultados contrastan con lo observado en *S. senegalensis*, pues, si bien se demuestra que la expresión de los genes *fads2* y *elovl5* no varía en función del contenido en PUFA n-3 de la dieta para larvas en estadio temprano (Capítulo 4; Torres *et al*., 2020a), no ocurre lo mismo en estadios posteriores (larvas tardías y poslarvas). De forma paradójica, *fads2* muestra un patrón de expresión opuesto en larvas tardías y poslarvas de *S. senegalensis* (Capítulo 4 y 5; Torres *et al*., 2020a, b). Así, la expresión de *fads2* es regulada el alza en las vísceras y en la cabeza de las larvas tardías alimentadas con la dieta más pobre en PUFA n-3 (Capítulo 4; Torres *et al*., 2020a), mientras que dicha sobreexpresión se produce únicamente en las vísceras de las poslarvas alimentadas durante 15 días con la dieta más rica en PUFA n-3 (Capítulo 5; Torres *et al*., 2020b). Curiosamente, estas diferencias en el patrón de expresión visceral no se observan en las poslarvas tras 30 días de tratamiento dietario (Capítulo 5; Torres *et al*., 2020b). Por tanto, estos datos sugieren que la expresión visceral de *fads2* en lenguado podría depender del tiempo de tratamiento dietario, aunque se necesitarían llevar a cabo ensayos adicionales para su confirmación. Además, estas diferencias podrían ser consecuencia de la síntesis localizada y posterior movilización de los ácidos grasos desde el sistema digestivo hacia otros

tejidos para responder adecuadamente a los cambios fisiológicos ocurridos durante el periodo de estudio.

En contraste, la expresión de *elovl5* se mantiene al alza en las vísceras de aquellas poslarvas de lenguado alimentadas durante 30 días con la dieta más rica en PUFA n-3. Estos resultados, no solo evidencian la manera independiente en que *fads2* y *elovl5*, ambos involucrados en la síntesis de LC-PUFA, responden ante un bajo contenido dietario de sus precursores, sino que, además, resaltan la manera específica en que ambas Fads2 (Δ4 y Δ6), con actividad desaturasa sobre sustratos distintos, operan en la ruta de biosíntesis de DHA. Así, la regulación positiva de la actividad desaturasa Δ4, observada tanto en la región visceral como en la cabeza de las larvas de *S. senegalensis* en respuesta a una dieta más baja en PUFA n-3, podría garantizar que los niveles de DHA permanezcan constantes ante una ingesta limitada de este compuesto en la dieta (Kuah *et al*., 2015; Morais *et al*., 2012). Esto podría ser indicativo de la importancia de la producción de DHA, a partir de EPA, a través de la denominada vía Δ4 para mantener una reserva óptima de DHA durante el proceso de neurogénesis, así como el posterior desarrollo y funcionalidad neuronal (Turkmen *et al*., 2017). De igual manera, los datos sugieren la importancia biológica de esta vía para reducir la dependencia alimentaria de LC-PUFA n-3 durante las primeras etapas del desarrollo del lenguado senegalés, en comparación con otros peces marinos como la dorada (Morais *et al*., 2012; Turkmen *et al*., 2017). Este hecho podría explicar la

baja mortalidad que esta especie, en contraste con otras, experimenta ante el uso de presas vivas sin enriquecer como alimento durante su desarrollo larvario (Morais *et al*., 2004; Villalta *et al*., 2005).

Asimismo, las diferencias observadas en el patrón de expresión de *fads2* y *elovl5* entre larvas tardías y poslarvas de lenguado podrían ser resultado de la competencia existente entre los PUFA n-3 y n-6 como sustratos en el metabolismo anabólico de los respectivos LC-PUFA. Esto es debido a que la conversión de ácidos grasos de la serie n-3 y n-6 comparte la misma maquinaria enzimática, es decir los PUFA n-3 y n-6 compiten por actuar como sustratos de la Fads2 y de la Elovl5 en la ruta biosintética de LC-PUFA (**Figura 3.1**). Por tanto, un exceso de ácidos grasos de una serie puede causar una disminución significativa en la conversión de los ácidos grasos de la otra (Schmitz y Ecker, 2008). Esto podría ocurrir en el caso de *S. senegalensis*, donde un alto aporte dietario en LC-PUFA n-6 durante el desarrollo larvario puede tener efectos negativos sobre su morfogénesis y pigmentación (Boglino *et al*., 2014). Además, una incorrecta relación entre el contenido en LC-PUFA n-3 y n-6 en la dieta, o incluso niveles subóptimos de ARA, puede afectar al metabolismo lipídico, haciendo que la actividad de sus enzimas Fads2 y Elovl5 sean moduladas, dirigiendo su actividad hacia la biosíntesis de LC-PUFA n-6, en detrimento de la síntesis de EPA o DHA (Norambuena *et al*., 2013).

Igualmente, estas diferencias observadas en el patrón de expresión de *fads2* y *elovl5*, ya sea entre ambas especies o entre diferentes estadios de la misma, podrían deberse a múltiples factores como la ya comentada anteriormente actividad específica de la desaturasa Fads2, la diferente edad fisiológica de los peces empleados en los experimentos, o las posibles diferencias en la cantidad de alimento ingerido por ambas especies durante el período de ensayo.

Ya centrados en el análisis de los resultados referentes a la regulación nutricional de los genes *elovl4a* y *elovl4b* en ambas especies a lo largo del desarrollo larvario/poslarvario, se observa que en larvas tempranas únicamente la expresión de *elovl4a* es sobrerregulada en respuesta a una dieta rica en PUFA n-3 (Capítulo 4; Torres *et al*., 2020a). Curiosamente, en larvas tardías de 40 dpe sólo *elovl4b* se expresa al alza en peces alimentados con la dieta enriquecida en PUFA n-3 (Capítulo 4; Torres *et al*., 2020a). En contraste, a excepción de lo observado en la cabeza de lenguado, la expresión de *elovl4a* y *elovl4b* se ve aumentada en poslarvas alimentadas durante 30 días con una dieta más pobre en LC-PUFA (Capítulo 5; Torres *et al*., 2020b). De esta manera, el patrón de expresión de *elovl4a* y *elovl4b* en poslarvas de ambas de especies parece responder de manera similar a lo observado en otros teleósteos, en los que una dieta con un bajo contenido en LC-PUFA n-3 da lugar a la sobreexpresión de *elovl4* (Li *et al*., 2017a, b; Yan *et al*., 2018; Zhao *et al*., 2019). Esto podría ser indicativo de que, a diferencia de lo observado durante el

periodo larvario, en la fase poslarvaria, un contenido dietario deficiente en LC-PUFA n-3 podría iniciar la activación de un mecanismo compensatorio, inicialmente definido por un aumento de la transcripción de los genes *elovl4*, que permitiría contrarrestar la falta de sustratos disponibles para la biosíntesis de los VLC-PUFA.

Estas diferencias observadas en el patrón de expresión de *elovl4a* y *elovl4b* en respuesta al contenido en ácidos grasos de la dieta a lo largo de las diferentes etapas del desarrollo larvario, durante las que ambas especies son alimentadas con presas vivas, y poslarvario, en las que son alimentadas con dietas inertes, podrían atender a las diferentes demandas de PUFA (LC- y VLC-PUFA) a las que *S. aurata* y *S. senegalensis* se enfrentan en función de la etapa y el grado de desarrollo fisiológico. Por lo tanto, basándonos en la funcionalidad descrita de Elovl4a y Elovl4b para *S. aurata* y *S. senegalensis*, así como en el patrón diferencial de expresión tisular observado, caracterizado por una expresión mayoritaria de *elovl4a* en el cerebro (Jin *et al*., 2017; Monroig *et al*., 2010; Oboh *et al*., 2017b; Zhao *et al*., 2019) y de *elovl4b* en los ojos (Betancor *et al*., 2020; Carmona-Antoñanzas *et al*., 2011; Jin *et al*., 2017; Li *et al*., 2017b; Monroig *et al*., 2010; Zhao *et al*., 2019), podemos vincular el aumento en la expresión de *elovl4a* y *elovl4b* a la demanda específica de VLC-SFA y VLC-PUFA que se produce durante el desarrollo del sistema nervioso de ambas especies, cuya síntesis está predominantemente asociada a la actividad Elov4a y Elov4b, respectivamente.

Por lo tanto, a pesar de que la función fisiológica de las diferentes moléculas de VLC-FA aún no ha sido perfectamente definida y que su escasa presencia dificulta en gran medida su análisis e identificación en peces (Garlito *et al*., 2019; Monroig *et al*., 2016), la interpretación de los resultados aquí expuestos, referentes al patrón de expresión de *elovl4a* y *elovl4b* en ambas especies, sugiere un papel relevante de estas enzimas en la biosíntesis y la incorporación localizada de los VLC-SFA y VLC-PUFA en los tejidos neurales de los peces. Estos resultados coinciden con la funcionalidad descrita en mamíferos (Deák *et al*., 2019), en los que los VLC-FA son componentes de vital importancia para el desarrollo y la protección de las células que conforman los tejidos neurales (Agbaga *et al*., 2008, 2010; Bazan, 2018). Más específicamente, ciertos VLC-PUFA se sintetizan y esterifican en la posición *sn-1* del esqueleto de glicerol de la fosfatidilcolina (Agbaga *et al*., 2010; Deák *et al*., 2019), para ser depositados posteriormente en los fotorreceptores de la retina donde juegan un importante papel neuroprotector (Bennett *et al*., 2014; Deák *et al*., 2019). De igual manera, los VLC-SFA son incorporados principalmente en forma de esfingolípidos dentro del sistema nervioso central (Deák *et al*., 2019), desempeñando un papel clave en la fusión de la membrana de las vesículas sinápticas que se produce durante el proceso de neurotransmisión (Hopiavuori *et al*., 2018, 2019). Por lo tanto, una correcta estimulación de la expresión de los genes *elovl4* a través de la dieta, que podría llevar asociado un aumento en la transcripción

de las proteínas que codifican y una mayor biosíntesis de sus productos asociados, puede ser de vital importancia para el correcto desarrollo y función de la estructura neural y visual durante las primeras fases del desarrollo de los peces. Así, aunque estas estructuras cognitivas no están inicialmente desarrolladas en las larvas recién eclosionadas, los conos y los batones retinianos se diferencian rápidamente (Hu *et al*., 2018; Lim *et al*., 2014). Este rápido proceso de retinogénesis permite que las larvas de los peces puedan comenzar a alimentarse activamente de manera exógena (Turkmen *et al*., 2017). Por tanto, se podría deducir que el buen desarrollo y función de las estructuras retinianas es un factor determinante para la supervivencia de las especies con hábitos de depredación visual, como son la dorada y el lenguado senegalés.

Finalmente, cabe resaltar que la aplicación de estos conocimientos tiene una especial relevancia en el cultivo de especies marinas con alto interés comercial para la producción acuícola, y que debe tenerse en cuenta en el diseño de los protocolos de alimentación durante su cría. Asimismo, estos resultados pueden ayudar a dilucidar el mecanismo molecular que controla la biosíntesis de los VLC-PUFA y sus requerimientos específicos en cada especie durante el desarrollo de los peces marinos. Además, proporcionan una valiosa información a tener en cuenta para la formulación y el diseño de dietas dirigidas a las primeras fases del desarrollo de los peces cultivados. Con ello, se abre la posibilidad de reducir la utilización de los

"ingredientes marinos", es decir aceites y harinas de pescado, cada vez más escasos y controvertidos, para poder incorporar con éxito fuentes alternativas de lípidos, mediante una programación nutricional temprana que estimule la biosíntesis de los LC-PUFA y los VLC-PUFA durante las primeras etapas del desarrollo y, por ende, durante el inicio de su alimentación exógena.

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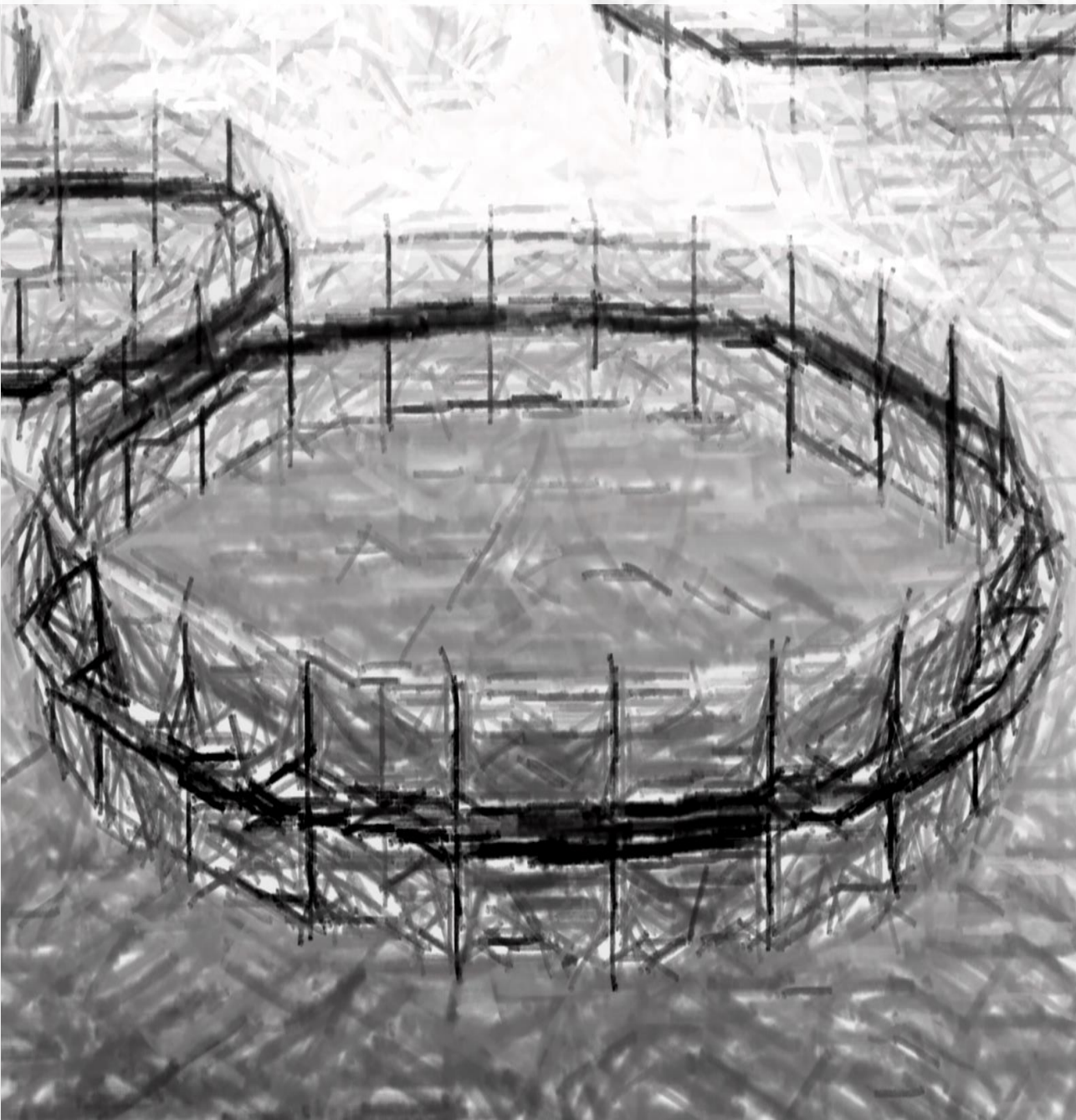
CONCLUSIONES



1. Los genes de elongación de ácidos grasos *elovl4a* y *elovl4b* están presentes y son funcionales en el genoma de la dorada (*Sparus aurata*) y el lenguado senegalés (*Solea senegalensis*).

The fatty acid elongation genes *elovl4a* and *elovl4b* are present and functional in the genome of gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*).

2. Aunque con algunas diferencias, las enzimas Elovl4a y Elovl4b participan en la biosíntesis de VLC-SFA y VLC-PUFA de hasta C_{34} a partir de una amplia variedad de sustratos en ambas especies.

Although with some specificities, Elovl4a and Elovl4b are involved in the biosynthesis of VLC-SFA and VLC-PUFA up to C_{34} being able to elongate a range of substrates in both species.

3. La expresión de los genes *elovl4* está mayoritariamente localizada en los tejidos neurales. Concretamente, los ojos y el cerebro muestran los mayores valores de expresiones de *elovl4a* y *elovl4b*, respectivamente.

Neural tissues are the major site of *elovl4* expression. Specifically, the brain and eye show the highest *elovl4a* and *elovl4b* expression levels, respectively.

4. La presencia de transcritos ARNm de *elovl4a* y *elovl4b* en embriones y larvas de los peces, fase de huevo incluida, sugiere la importancia de una correcta biosíntesis de VLC-FA durante el desarrollo temprano o fase de alimentación endógena en ambas especies .

The presence of *elovl4a* and *elovl4b* mRNA transcripts in embryos and larval fish, including the eggs before hatching, suggests that VLC-PUFA biosynthesis can be important in early development or endogenous feeding stage of both species.

5. Existe relación entre el periodo de retinogénesis y el patrón de expresión de los genes *elovl4* en ambas especies. Este hecho puede ser indicativo de la importancia que los VLC-PUFA desempeñan para el correcto desarrollo de la visión durante las etapas iniciales de la dorada y el lenguado senegalés.

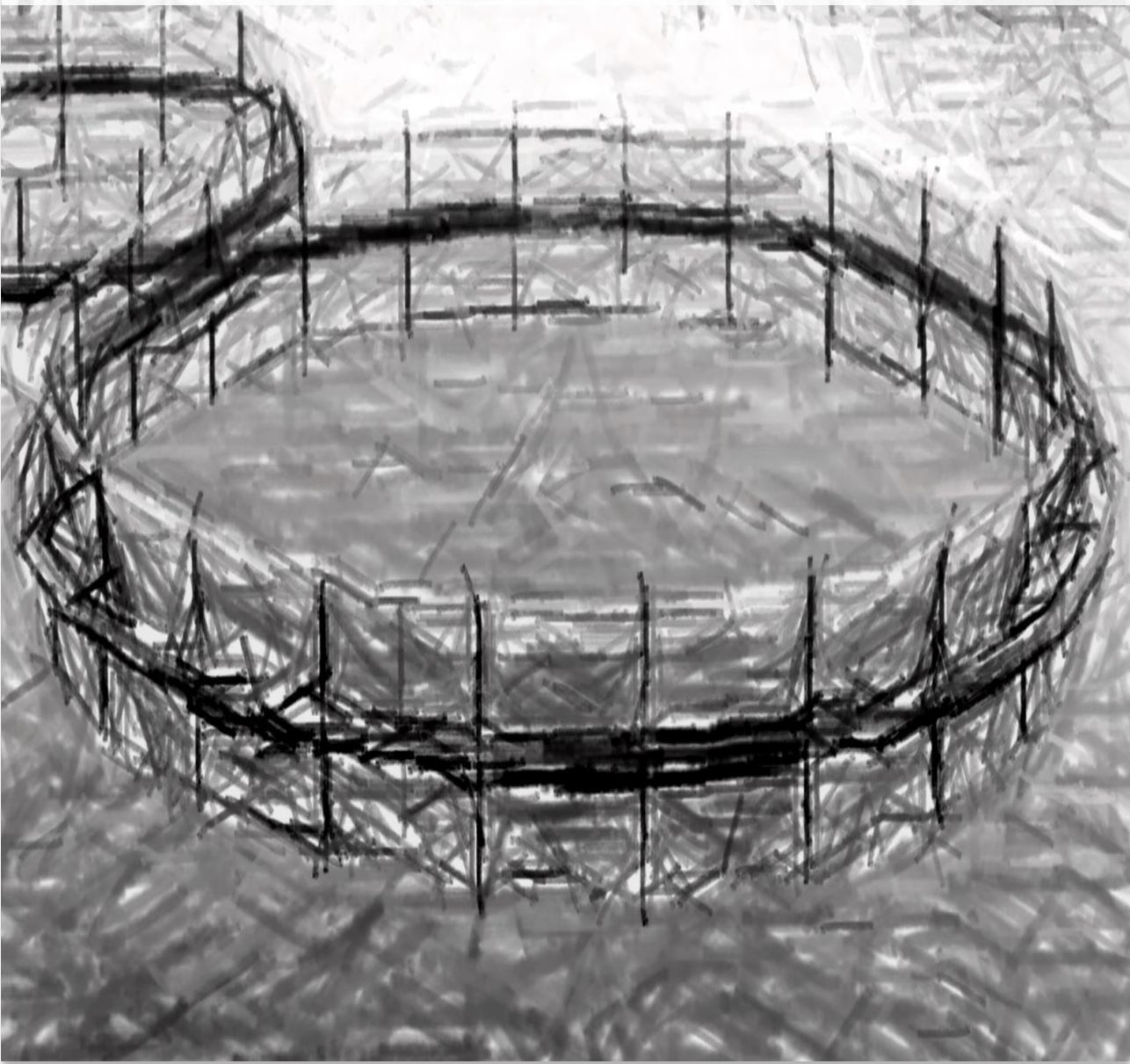
There is a synchrony between the timing at which retinogenesis occurs in both species and an increased expression of the two *elovl4* genes. This could be demonstrative of the importance of VLC-PUFA for the correct

development of vision during early larval development of gilthead seabream and Senegalese sole.

6. La expresión de los genes codificantes para las enzimas Fads2, Elovl5, Elovl4a y Elovl4b puede ser regulada a través del contenido dietario en LC-PUFA. Además, la regulación nutricional de los genes *elovl4* es específica de cada especie, atendiendo a las diferentes necesidades de VLC-PUFA asociadas a cada etapa de desarrollo.

Transcription of *fads2*, *elovl5* and *elovl4* genes can be regulated by dietary LC-PUFA. Moreover, nutritional regulation of *elovl4* is species-specific, depending on the VLC-PUFA needs associated with each life-stage.

RESÚMENES



Very long-chain fatty acids (VLC-FA; $>C_{24}$), although present in small amounts, play important roles for the correct development and functionality of neural tissues, especially during early development of vertebrates. However, despite their putative importance, their study in fish is scarce.

Biosynthesis of VLC-FA is carried out by the so-called elongation of very long-chain fatty acid 4 (Elovl4) proteins and, consequently, the complement and function of these enzymes determine the endogenous capacity that a given species has for satisfying the physiological demands for VLC-FA, especially during its early development. Moreover, this endogenous production of very long-chain polyunsaturated fatty acid (VLC-PUFA) is substrate-dependent. Therefore, shorter fatty acid precursors, i.e. long-chain polyunsaturated fatty acids (LC-PUFA; C_{20-24}), are required. These nutrients are mostly incorporated by the diet and their bioavailability can influence the capacity of Elovl4 for satisfying the physiological VLC-PUFA demands in marine fish. Thus, nutritional regulation of *elovl4*, as well as other elongase and desaturase genes involved in LC-PUFA biosynthesis (*elovl5*, *fads2*) has been proposed as a strategy to enhance endogenous production of LC-PUFA and VLC-PUFA in fish farming.

The present thesis aimed to characterize *elovl4* genes from the marine teleosts *Sparus aurata* and *Solea senegalensis*, to determine the function of the corresponding encoded proteins, and to analyze the tissue expression pattern of these

genes. Moreover, we investigated the nutritional regulation of genes involved in the biosynthesis of the VLC-PUFA (*elovl4a*, *elovl4b*) and the LC-PUFA (*fads2*, *elovl5*) in early life-cycle stages (larvae and post-larvae) of both fish species fed diets adapted to each development stage. Live preys were supplied to larvae (early and late larvae) and microdiets for post-larvae, with a variable content in VLC-PUFA precursors, i.e. LC-PUFA.

The results confirmed that both fish species possess two distinct *elovl4* genes termed as *elovl4a* and *elovl4b* based on their homology to the zebrafish orthologs. Functional assays of the corresponding proteins in yeast denoted that Elovl4a and Elovl4b from both species have the capability to elongate C_{20-24} fatty acid precursors to VLC-FA products. However, Elovl4b appeared to have a higher activity than Elovl4a elongating all the polyunsaturated fatty acid substrates assayed to longer chain polyunsaturated products, especially those of the n-3 series. Moreover, gene expression results indicated that, although *elovl4* transcripts were detected in most of the tissues analyzed, *elovl4* genes were more strongly expressed in the neural tissues of both species, such as brain and eyes, which showed the highest expression levels of *elovl4a* and *elovl4b*, respectively. Furthermore, the results from nutritional regulation assays denoted that *fads2*, *elovl5*, *elovl4a* and *elovl4b* genes could be regulated by the dietary LC-PUFA content. It is important to highlight that *elovl4a* and *elovl4b* genes were differently regulated according to the species-specific VLC-

PUFA putative needs, associated with each early life-stage and the LC-PUFA dietary availability.

Importantly, these findings can contribute to a better understanding of the VLC-FA biosynthetic pathway in marine teleosts, highlighting the crucial role that the Elovl4 products carry out for the correct development and maintenance of neurophysiological functions during early stages of the fish development. Therefore, these results can help to elucidate the molecular mechanism controlling the VLC-PUFA biosynthesis and their species-specific requirements along the marine fish development, opening the possibility to incorporate successfully alternative lipid sources, through an early nutritional programming that stimulates the VLC-PUFA biosynthesis during the first exogenous feeding stages.

Los ácidos grasos de cadena muy larga (VLC-FA; $>C_{24}$), aunque presentes en pequeñas cantidades, juegan un importante papel para el correcto desarrollo y funcionalidad de los tejidos neurales en vertebrados, especialmente durante su desarrollo temprano. Sin embargo, a pesar de su aparente importancia, los estudios sobre estos compuestos en peces son escasos.

La biosíntesis de los VLC-FA se lleva a cabo mediante las denominadas proteínas de elongación de los ácidos grasos de cadena muy larga 4 (Elovl4) y, en consecuencia, la dotación y la función de estas enzimas determinan la capacidad endógena que una determinada especie tiene para satisfacer las demandas fisiológicas de VLC-FA, especialmente durante su desarrollo temprano. Además, esta producción endógena de los ácidos grasos poliinsaturados de cadena muy larga (VLC-PUFA) es dependiente de los sustratos. Así, para su biosíntesis se requiere de ácidos grasos más cortos, es decir ácidos grasos poliinsaturados de cadena larga (LC-PUFA; C_{20-24}) que actúen como precursores. Estos nutrientes son incorporados principalmente a través de la dieta y su biodisponibilidad puede determinar la capacidad que las Elovl4 tienen para satisfacer las demandas fisiológicas de VLC-PUFA en peces marinos. Por lo tanto, la regulación nutricional de *elovl4*, así como la de otros genes elongasa y desaturasa involucrados en la biosíntesis de LC-PUFA (*elovl5, fads2*), ha sido propuesta como una estrategia para mejorar la producción endógena de LC-PUFA y VLC-PUFA en las especies marinas cultivadas.

Teniendo esto en mente, el presente trabajo de investigación tuvo como objetivos caracterizar los genes *elovl4* en dorada (*Sparus aurata*) y en lenguado senegalés (*Solea senegalensis*), determinar la función de sus correspondientes proteínas codificadas, así como analizar el patrón de expresión tisular de *elovl4*. Además, se ha investigado la regulación nutricional de los genes implicados en la biosíntesis de VLC-PUFA (*elovl4a*, *elovl4b*) y LC-PUFA (*fads2*, e*lovl5*) durante las primeras etapas del ciclo de vida de ambas especies (larvas y poslarvas), mediante el uso de dietas adaptadas a cada etapa del desarrollo. Se suministraron presas vivas durante el estado larvario (larvas tempranas y tardías) y microdietas inertes durante el estado poslarvario con un contenido variable en LC-PUFA, como precursores de los VLC-PUFA.

Los resultados confirmaron que ambas especies de peces poseen dos genes *elovl4* distintos, denominados *elovl4a* y *elovl4b* según su homología con sus ortólogos de pez cebra. Asimismo, los ensayos funcionales de sus correspondientes proteínas, llevados a cabo en levaduras, indicaron que tanto Elovl4a como Elovl4b tienen la capacidad de elongar los ácidos grasos precursores (C_{20-24}) hasta VLC-FA en ambas especies. Sin embargo, Elovl4b mostró mayor actividad que Elovl4a para elongar todos los ácidos grasos poliinsaturados a productos de cadena más larga, especialmente de la serie n-3. Además, los resultados de expresión génica indicaron que, aunque fueron detectados transcritos de *elovl4* en la mayoría de los tejidos

analizados, ambos genes *elovl4* se expresaron más intensamente en los tejidos neurales de ambas especies, como el cerebro y los ojos, que mostraron los niveles de expresión más altos de *elovl4a* y *elovl4b*, respectivamente. Además, los resultados procedentes de los ensayos de regulación nutricional indicaron que los genes *fads2, elovl5, elovl4a* y *elovl4b* pueden ser regulados a través del contenido en LC-PUFA presente en la dieta. Es importante destacar que *elovl4a* y *elovl4b* fueron regulados de manera distinta según las hipotéticas necesidades de VLC-PUFA asociadas, de manera específica, con cada etapa del desarrollo temprano y dependiendo de disponibilidad dietaria de LC-PUFA.

Estos hallazgos pueden contribuir a alcanzar una mejor comprensión de la vía biosintética de los VLC-FA en los teleósteos marinos, resaltando así el papel crucial que los productos de Elovl4 desempeñan para el correcto desarrollo y mantenimiento de las funciones neurofisiológicas durante las primeras etapas del desarrollo de los peces. Asimismo, estos resultados pueden ayudar a esclarecer el mecanismo molecular que controla la biosíntesis de VLC-PUFA, así como a establecer sus requerimientos específicos a lo largo del desarrollo de los teleosteos marinos en función de la especie. De esta manera, se abre la posibilidad de incorporar con éxito fuentes lipídicas alternativas a través de una programación nutricional temprana que estimule la biosíntesis de los VLC-PUFA durante las primeras etapas de alimentación exógena.

Els àcids grassos de cadena molt llarga (VLC-FA; $>C_{24}$), encara que presents en petites quantitats, juguen un important paper per al correcte desenvolupament i funcionalitat dels teixits neurals en vertebrats, especialment durant el seu desenvolupament inicial. No obstant, malgrat la seua aparent importància, els estudis sobre aquests compostos en peixos son escassos.

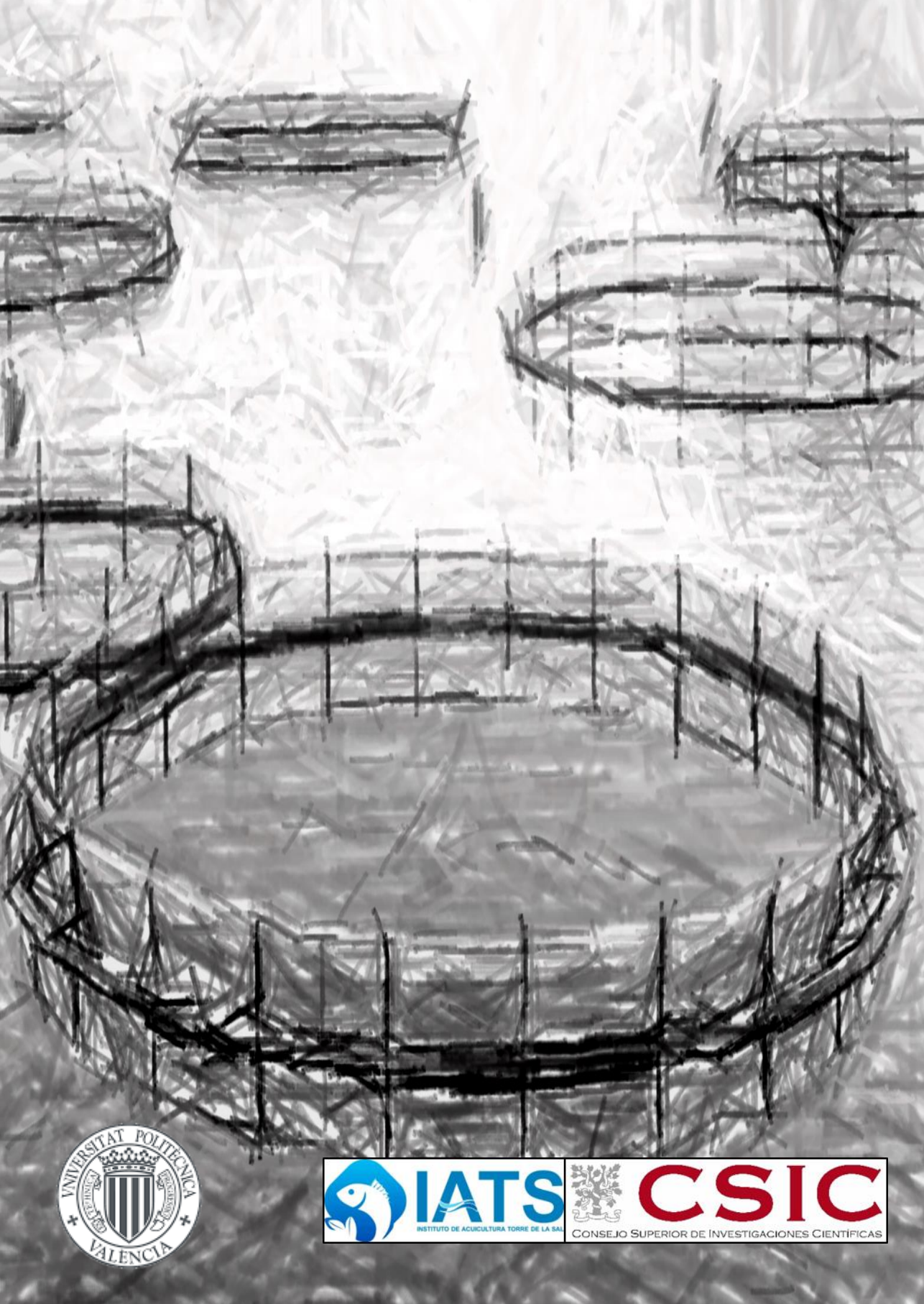
La biosíntesi dels VLC-FA es porta a terme mitjançant les denominades proteïnes d'elongació dels àcids grassos de cadena molt llarga 4 (Elovl4) i, en conseqüència, la dotació i la funció d'aquests enzims determinen la capacitat endògena que una determinada espècie té per a satisfer les demandes fisiològiques de VLC-FA, especialment durant el seu desenvolupament inicial. A més, aquesta producció endògena dels àcids grassos poliinsaturats de cadena molt llarga (VLC-PUFA) és depenent dels substrats. Així, per a la seua biosíntesi es requereix d'àcids grassos més curts, és a dir àcids grassos poliinsaturats de cadena llarga (LC-PUFA; C_{20-24}) que actuen com a precursors. Aquests nutrients són incorporats principalment a través de la dieta i la seua biodisponibilitat pot determinar la capacitat que les Elovl4 tenen per a satisfer les demandes fisiològiques de VLC-PUFA en peixos marins. Per tant, la regulació nutricional de *elovl4*, així com la d'altres gens elongasa i desaturasa involucrats en la biosíntesi de LC-PUFA (*elovl5*, *fads2*), ha estat proposada com una estratègia per a millorar la producció endògena de LC-PUFA i VLC-PUFA en les espècies marines cultivades.

Tenint això en compte, el present treball de recerca va tindre com a objectius caracteritzar els gens *elovl4* en orada (*Sparus aurata*) i llenguado (*Solea senegalensis*), determinar la funció de les seues corresponents proteïnes codificades, així com analitzar el patró d'expressió tissular de *elovl4*. A més, es va investigar la regulació nutricional dels gens implicats en la biosíntesi de VLC-PUFA (*elovl4a*, *elovl4b*) i LC-PUFA (*fads2*, *elovl5*), durant les primeres etapes del cicle de vida d'ambdues espècies (larves i poslarves), mitjançant l'ús de dietes adaptades a cada etapa del desenvolupament. Es subministraren preses vives durant l'estadi larvari (larves primerenques i tardanes) i microdietes inerts durant l'estadi poslarvari amb un contingut variable en LC-PUFA, com precursors dels VLC-PUFA.

Els resultats van confirmar que totes dues espècies de peixos posseeixen dos gens *elovl4* diferents, denominats *elovl4a* i *elovl4b* segons la seua homologia amb els seus ortòlegs en peix zebra. Així mateix, els assajos funcionals de les corresponents proteïnes, duts a terme en llevats, van indicar que tant Elovl4a com Elovl4b tenen la capacitat d'elongar els àcids grassos precursors (C_{20-24}) fins a VLC-FA en totes dues espècies. No obstant això, Elovl4b va mostrar major activitat que Elovl4a per a elongar tots els àcids grassos poliinsaturats (substrats) fins a productes de cadena més llarga, especialment de la sèrie n-3. A més, els resultats d'expressió gènica van indicar que, encara que van ser detectats trànscrits de *elovl4* en la majoria dels teixits analitzats, tots dos gens *elovl4* es van expressar més en teixits neurals de totes dues

espècies, com el cervell i els ulls, que van mostrar els nivells d'expressió més alts d'*elovl4a* i *elovl4b*, respectivament. A més, els resultats procedents dels assajos de regulació nutricional van indicar que els gens *fads2*, *elovl5*, *elovl4a* i *elovl4b* poden ser regulats a través del contingut en LC-PUFA present en la dieta. És important destacar que *elovl4a* i *elovl4b* van ser regulats de manera diferent segons les hipotètiques necessitats de VLC-PUFA associades, de manera específica, amb cada etapa del desenvolupament inicial i depenent de la disponibilitat dietaria de LC-PUFA.

Aquestes troballes poden contribuir a tenir una millor comprensió de la via biosintètica dels VLC-FA en els teleostis marins, ressaltant així el paper crucial que els productes d'Elovl4 exerceixen en el correcte desenvolupament i manteniment de les funcions neurofisiològiques durant les primeres etapes del desenvolupament dels peixos. De la mateixa forma, aquests resultats poden ajudar a esclarir el mecanisme molecular que controla la síntesi endògena de VLC-PUFA, així com a establir els requisits específics de cada espècie al llarg del desenvolupament dels teleostis marins. D'aquesta manera, s'obri la possibilitat d'incorporar amb èxit fonts lipídiques alternatives a través d'una programació nutricional inicial que estimuli la biosíntesi de VLC-PUFA durant les primeres etapes d'alimentació exògena.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